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CONTENTS

PAGE

Histochemical Characterization of Wheat and Wheat Products. I. Histochemical Demonstration of Germ and Aleurone Using Acridine Orange. *Y. Pomeranz and J. A. Shellenberger* 103

Histochemical Characterization of Wheat and Wheat Products. II. Mapping of Protein Distribution in the Wheat Kernel. *Y. Pomeranz and J. A. Shellenberger* 109

Histochemical Characterization of Wheat and Wheat Products. III. Use of Methyl Green in Estimating Flour Extraction Rate. *Y. Pomeranz and J. A. Shellenberger* 113

Histochemical Characterization of Wheat and Wheat Products. IV. Mapping the Free Fatty Acids in Germinating Wheat. *Y. Pomeranz and J. A. Shellenberger* 122

Histochemical Characterization of Wheat and Wheat Products. V. Sulfhydryl Groups: Their Localization in the Wheat Kernel. *Y. Pomeranz and J. A. Shellenberger* 133

The Formation of Dough and Bread Structures. I. The Ability of Starch to Form Structures, and the Improving Effect of Glyceryl Monostearate. *G. Jongh* 140

A Farinograph Technique for Macaroni Doughs. *G. N. Irvine, J. W. Bradley, and G. C. Martin* 153

Some Volatile Carbonyl Compounds Arising during Panary Fermentation. *F. E. Kohn, L. Wiseblatt, and L. S. Fosdick* 165

The Control of Fungi during the Malting of Wheat. *James R. Fleming, John A. Johnson, and Byron S. Miller* 170

The Bromate Reaction in Dough. III. Effect of Continuous Mixing and Flour Particle Size. *W. Bushuk and I. Hlynka* 178

Zone Electrophoresis Pattern of Free Amino Acids As an Index of Storage Condition of Wheat. *Pekka Linko* 187

A Note on Self-Diffusion in Dough Containing Br^{82}-Labeled Bromate. *C. C. Lee and R. Tkachuk* 194

A Note on a Rapid Method for the Determination of Lipids in Brewing Adjunct Cereals. *Gerhard J. Haas and Alan I. Fleischman* 198

Note on a Simplified Rapid Method for Determining Alpha-Amylase Activity. *Sven Hagberg* 202

A Note on the Construction of a Small-Scale Dough Expansion Apparatus for the Estimation of Wheat Quality. *H. Miller, A. G. O. Whiteside, and W. Kalbfleisch* 204

Editorial Policy and Suggestions to Authors 206

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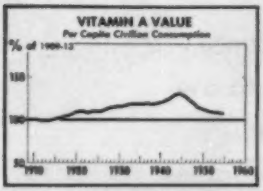
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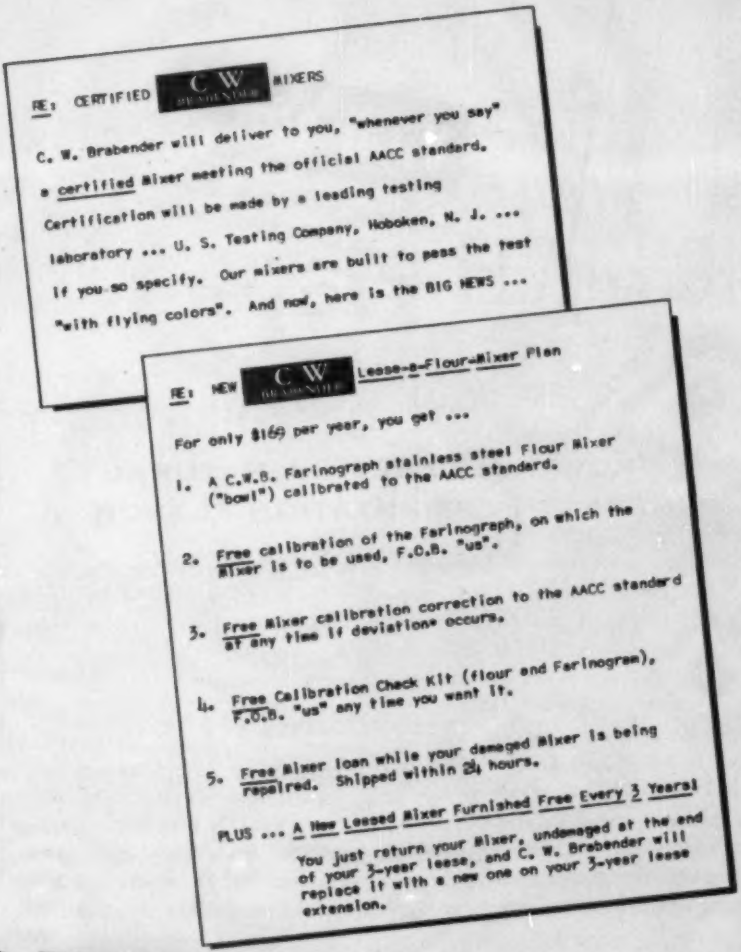
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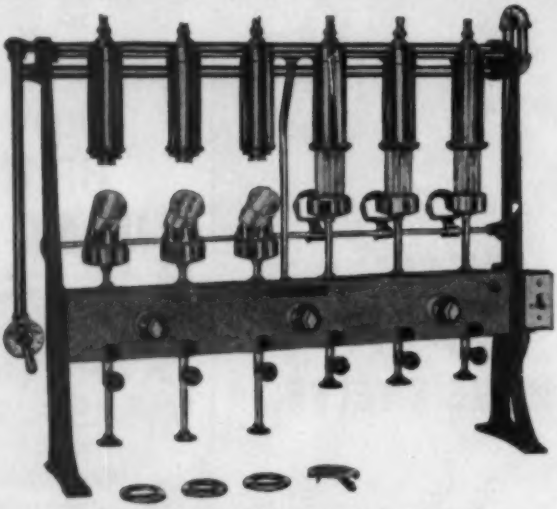
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CEREAL CHEMISTRY

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HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

I. Histochemical Demonstration of Germ and Aleurone Using Acridine Orange[1]

Y. POMERANZ AND J. A. SHELLENBERGER

ABSTRACT

Polychromatic fluorescent properties of acridine orange solutions were used to characterize wheat tissues and milled wheat products. A water solution of the dye can be used for multicolor staining of wheat tissues. Coloration varies with protein content.

A saturated xylol (xylene) solution of the base forms a brilliant fluorescence with the lipid material of either the aleurone or the germ so it can be used to distinguish these two wheat tissues in milled products.

Methods available to characterize wheat tissues in flour are limited, although this subject is one of considerable practical concern to the milling industry. Methods used depend on gross differences in the chemical composition of the various tissues of the wheat kernel. Such methods are useful only when a certain constituent is present in relatively large amount in one tissue and essentially absent in another — and only when differences are detectable by sensitive and reproducible chemical tests. Therefore, a dye adsorption test that characterizes wheat tissue in milled products should prove useful.

Fat concentration of the wheat kernel is highest in the embryo and the aleurone layer (10). According to Meyer (9), 2% of the wheat kernel is oil, while the content of lipids in the bran and the germ is 5–6% and 12–18%, respectively. Combined germ and aleurone content of a milled wheat product can be estimated from its oil content. However, the technique of determining the amount of lipid-rich tissue particles in ground flour depends on a number of factors such as the fol-

[1] Manuscript received April 23, 1960. Presented at the 45th annual meeting, Chicago, Illinois, May 1960. Contribution No. 333, Department of Flour and Feed Milling Industries, Kansas State University Agricultural Experiment Station, Manhattan; part of a thesis submitted to the Graduate School of Kansas State University by Y. Pomeranz, in partial fulfillment of the requirements of the Ph.D. degree.

lowing, which cause variations in the oil content of flour:

a) Tempering and milling procedures influence the behavior of the endosperm, pericarp, aleurone, and germ from various wheats (5,6).

b) Oil may be pressed from the germ during milling and so oil in the germ may decrease while it increases in the flour (12).

c) The analytical procedure to determine fat is not precise enough to detect minute differences of lipid-rich material in flour (10).

The difficulty of estimating, on the basis of fat content, the particles in flour originating from germ and aleurone tissues led to experiments with histochemical methods.

Two methods using acridine orange (AO) were investigated. First, a slightly acid water solution of the dye was used according to staining schedules recommended in the literature for animal tissues. A second procedure employed a saturated xylol (mixture of xylenes) solution of the base of the dye.

Materials

Materials used were:

1. Longitudinal and transectional slices prepared from kernels of soft and hard winter wheat by the procedure of Grosh and Milner (4).

2. Various grades of commercially milled flour and samples of highly refined white flour with various amounts of purified and ground germ added. (Germ used, a commercial product, had extraneous material removed by hand under low magnification.) A whole-wheat meal ground to pass a 40-mesh sieve was also prepared.

Methods

Experiments were carried out with AO, a xanthene dye (C.I. No. 788; Synonyms: Basic Orange 3 RN, Euchrysine 3 RXA). The fluorochrome (2) dye, whose purity and percentage of active compound were unknown, was obtained from Coleman & Bell Co., Norwood, Ohio.

The dye base was prepared by the following procedure outlined by Knaysi for preparation of the base of neutral red (7): 0.2 g. of dye were dissolved in 100 ml. water and filtered; the dye base was precipitated with *N* sodium hydroxide solution, and an excess of 1 to 2 drops of the alkali was added. The base precipitates imediately and can be filtered. The precipitate was washed with slightly alkaline water (pH 8.0) and, after air-drying, was kept in a stoppered glass bottle. The precipitated dye base remained stable for months.

A saturated solution of the dye base in xylol was prepared by shaking a 0.1% suspension for 15 minutes and then filtering. A shift

in color may take place after the dye base has stood for a time, so a fresh solution was prepared daily.

Staining with an Aqueous Solution of Acridine Orange (AO). This procedure was introduced in fluorescent microscopy by Bukatsch and Haitinger (1) and independently by Strugger (13) and has found wide use. The physico-chemical properties of the fluorescence were investigated by Zanker (16,17). Metcalf and Patton (8) discussed the use of ultraviolet illumination and fluorescent dyes, especially AO, and studied details of structure and physiological differences in biological materials. Emig (3) recommended AO to stain plant tissues because it stains them a fast clear brown or dark orange.

Many investigators have used AO in staining schedules in recent years. A partial list of references regarding acridine orange in staining animal tissues was given by Von Bertalanffy and Bickis (15).

AO dissolved in water showed a gradual change of the fluorescent color from green to red with intermediate orange when the concentration of the dye increased from 0.001 to 0.10%. Schümelfelder (14) found the red fluorescence to be due to the polymer of AO in stronger solutions. The intermediate colors of yellow and orange were due to different mixtures of a green fluorescent monomer and red fluorescent polymer cations. Scheibe and Eder (11) have reported similar results with AO bound in stained tissues.

The wheat sections were covered with a 1% acetic acid solution mixed with an equal amount of 0.01% solution of AO and stained for 5 minutes. The procedure was established after a series of tests in which the concentration of dye, time of staining, and concentration of acid were varied. Minor changes in the techniques do not affect the test, except that solution of dye concentrated to 0.1% diminished the effectiveness of clear tissue differentiation, and solutions diluted to 0.001% were rather ineffective. The stained wheat sections were observed with either ordinary light or ultraviolet radiation (having a predominant wave length of 3,660 Å) and with either low-power magnification or without visual aids.

Staining with a Saturated Xylol Solution of the Base of Acridine Orange. Free bases of several common basic dyes may have different colors from their salts or soaps. The dye bases are soluble in neutral fat, xylol, and alcohol but practically insoluble in water. The extent the color of a dye base solution shifts, in a fat solvent or fat, toward the color formed on interaction with free fatty acids was found to be stoichiometric. The color shift is due to intramolecular arrangement of the base upon soap formation, and the color is independent of the nature of the fatty acids.

Despite recommendations (7), experiments in the authors' laboratory, using the base of neutral red in staining wheat sections or wheat flour to detect free fatty acid-rich particles or layers, gave unsatisfactory results.

Excellent contrast was obtained when the AO base was employed. In addition to the differences in solubility and shift in color shown by many dye bases, AO exhibited pronounced polychromatic fluorescence, with variations of dye concentration in a stained tissue preparation.

Wheat sections were stained 5 minutes or longer (depending on the thickness of sections) by covering the material in the cavity of a spot plate with a xylol solution of the dye base. The stained material was blotted on filter paper and observed after about 5 minutes.

With sufficiently thin (about 20 microns) sections, the following procedure was used: Sections were placed on filter paper (Whatman No. 5) and at 1-minute intervals spotted three times with a drop of the dye base solution. The excess dye was adsorbed by the paper and the sections were transferred to a microscope slide for examination. No differentiation or counterstaining was needed.

Differences in color were seen immediately under ultraviolet radiation in the dark, but exposure to the light source radiation for about 10 minutes gave more brilliant colors.

To test wheat flour or ground wheat, a 0.25-g. sample was shaken for 15 minutes with 2 ml. of the dye base in xylol in a small test tube, then poured over filter paper (9-cm. Whatman No. 4) on a Büchner funnel and allowed to drain. The material could be tested on the filter paper after it was transferred to a Petri dish, allowed to dry (about 10 minutes), and observed under low magnification (24 or 48×, depending on particle size), employing a UV lamp as the source of radiation. The background of the filter paper, which shows a faint fluorescence, is not objectionable, but in case better contrast is desired the particles can be transferred, after drying, to a new sheet of paper.

Pictures were made with UV radiation on Ektachrome high-speed films.

Results and Discussion

Aqueous Solution of Acridine Orange. Viewed under ordinary light, both the pericarp layers and the germs, when stained with a water solution of AO, showed a rather intense dye uptake of yellow-brown. On examination of tissues exposed in the dark to a source of ultraviolet radiation, germ and pericarp showed (under 96×

magnification) the following multicolored fluorescent effect:

Scutellum (cotyledon) — bright yellow-green
Plumule and coleoptile — yellow-green
Aleurone — slight but distinct blue-green
Inner pericarp — orange-red
Outer pericarp — green-yellow
Starchy endosperm — unstained, light blue-violet background

The differences in color of the various tissues in the sectioned wheat kernel seemed to be due to both the selectivity and intensity of dye adsorption by the layers and the effect of polychromacy of the dye. Staining with AO gives contrasting colors that help distinguish the structural elements of tissues without applying the rather complicated techniques of multiple stains. The method eliminates difficulties due to fixation, dehydration, counterstaining, etc.; the low concentrations applied reduce the danger of artifacts (8).

To identify wheat tissues in a milled product, flour or wheat meal was tested also by the procedure given for the dye base method, employing the aqueous staining solution instead of the xylol base solution.

When the dry paper was observed under low magnification (24×), germ and aleurone particles showed strong yellow-green fluorescence, though the fluorescence was not as brilliant as in the tests using the xylol solution of the base.

Figures 1 to 4 appear at the end of Part IV, page 129.

Saturated Xylol Solution of the Base of Acridine Orange. Figures 1 and 2 are pictures obtained on cross and longitudinal sections under low-power magnification, using a predominant radiation of 3,660 Å. No effort was made to cut out the small amount of visible light, as it was desirable to see the background of the nonfluorescing wheat layers.

Although lipid material also is present to a certain extent in the endosperm and bran, it is only the germ and aleurone layer that show a distinct and brilliant red fluorescence due to the presence of fatty acids.

Figures 3 and 4 illustrate how easily the particles originating from the embryo or aleurone layer can be detected in ground whole wheat or milled flour. The figures were obtained under conditions similar to those outlined for wheat sections with the particles observed under higher magnification.

The aleurone and germ particles stand out clearly in the field, even though both the oil, mechanically exuded during milling, and other components, containing small concentrations of lipid materials, impart a faint pink shade.

Work to determine the extent of damage in wheat germs, as well

as lipase activity in cereal grains and microorganisms, is continuing, using the procedure for staining of wheat sections and lipid-containing material in milled wheat products.

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HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

II. Mapping of Protein Distribution in the Wheat Kernel[1]

Y. POMERANZ AND J. A. SHELLENBERGER

ABSTRACT

Topographical distribution of protein in wheat was followed on sections cut with a freezing microtome by means of the preferential adsorption of Xylidine Ponceau. The outer endosperm portions showed a higher dye-binding capacity than the central portions and the cheeks. Lowest protein concentration was in the pericarp; highest, in the aleurone layer and embryo.

Distribution of protein in the wheat kernel has attracted attention for many years. The recent separation of flour on a commercial basis to various products according to protein content has made protein distribution in the wheat kernel important.

Cobb (4) dissected individual kernels by hand and demonstrated a relatively sharp gradient in the protein content of the various endosperm zones. Morris *et al.* (13) dissected tempered wheat by using a dental drill and separated the endosperm into four fractions and the bran into two fractions. They found more protein in the peripheral endosperm zone than in the cheek, with the least in the central endosperm. The distribution of protein also has been studied in hand-dissected material by Hinton (9).

This report deals with the possibility of using a simple dye adsorption technique to map protein distribution in the wheat kernel. Various ways to demonstrate proteins by histochemical methods have been summarized by Serra (14), and more recently by Weiss *et al.* (17) and by Hopman (10). Burstone (3) discussed new approaches to the histochemical demonstration of proteins, based on condensation reactions in conjunction with coupling processes, or on oxidative deamination reactions. Although there are many tests for proteins, they are poorly adapted to histochemical work because they use strong acid or alkali which disintegrates cellular structure.

The method of Fraenkel and Cooper (6) to determine acidic and basic groups in proteins, based on the determination of unbound Orange G, was adapted by Udy (16) to estimate the protein content of ground wheat and flour, and by Bunyan (2) to evaluate organic nitrogen compounds in feed meals. Orange G gave yellow-brown

[1] Manuscript received April 23, 1960. Presented at the 45th annual meeting, Chicago, Illinois, May 1960. Contribution No. 334, Department of Flour and Feed Milling Industries, Kansas State University Agricultural Experiment Station, Manhattan.

shades that were rather unsuitable for microscopic techniques. The dye employed in this study, Xylidine Ponceau, was proposed by Levine (12) to stain basic amino groups.

Materials and Methods

Twenty-nine basic and acidic dyes recommended in the literature for protein staining were tested for localization and quantitative evaluation of protein in the wheat endosperm. Best results were obtained with Xylidine Ponceau 1 (5), Color Index 79, known also under several other synonyms. The acid, mono azo dye, has adsorption maxima at about 5,380 Å and 4,990 Å. It is water-soluble but has low solubility in 95% alcohol (about 0.2 g. in 100 ml.). Two series of tests were made to estimate the possible use of the dye for staining protein in wheat.

In one series, 67 samples of hard red winter wheat flour, milled on a Buhler experimental mill, were tested. The protein range of the flour samples was between 8.6 and 14.9%. The dye adsorption of the tested flours was determined by the following procedure: 2 g. of flour were suspended in 25 ml. of water containing 3 mg. of dye (using a 0.1% stock solution, diluted to the required strength) in a 125-ml. rubber-stoppered Erlenmeyer flask and shaken for 1 hour. After the material stood for 15 minutes, 15 ml. of the supernatant were transferred to a graduated tube and centrifuged until clear. (About 15 minutes at 2,000 r.p.m. (1,350 × g) was generally required.) The clear supernatant was diluted 1:2 and the absorbance measured with a Beckman model DU spectrophotometer within 4 hours from the beginning of the test. The solution developed opalescence after prolonged standing. The absorbance of solutions containing known amounts of dye obeyed the Beer-Lambert law in the range of concentrations used. Knowing the amount of unabsorbed dye permits the amount of bound dye to be calculated by difference.

In the second series of tests the dye adsorption of the following products was tested: a) commercial wheat starch; b) commercial wheat gluten containing 60% protein; c) pulverized coarse wheat bran; d) samples of commercial wheat germ, purified by removal of foreign material under low magnification (24×); e) wheat semolina; and f) wheat flour prepared by grinding of the semolina.

The dye-binding procedure was essentially the same as in series 1, except that the amount of tested material was reduced to 0.25 g. in samples c to f.

Based on findings in these two series of experiments, determinations of protein distribution were made on wheat sections 50 to 100

microns thick, prepared by the procedure of Grosh and Milner (8).

The sections were fixed overnight in 95% alcohol, kept for 1 minute in a 0.1% aqueous dye solution, washed in three changes of water, dehydrated by two changes of alcohol, mounted, and observed with the unaided eye or under low-power magnification. Photomicrographs were made with Kodachrome film.

The choice of the fixative should be governed by the chemical groups which serve for the visualization of the protein and the histochemical method employed (1). Alcohol has been found to be a rather unreactive fixative that causes the fewest changes in the structure of the protein and in available basic groups (15).

Results and Discussion

The correlation coefficient between the protein (as determined by the Kjeldahl method) and dye adsorption was +0.721 (significant at the 0.1% level) for the 67 experimentally milled flours.

The conditions under which the experimentally milled flours were tested for dye adsorption are not considered to be optimal for quantitative protein evaluation. Such a determination would involve use of more highly concentrated dye solutions. Because such solutions (i.e., 1%) gave poor contrast on stained wheat sections, a more dilute dye solution was employed.

Dye adsorption, under the described test condition, was 1.3 mg. per gram of wheat gluten, compared with 0.1 mg. per gram of wheat starch, confirming that the dye is bound mainly by endosperm proteins.

Figures 1 and 2 appear at the end of Part IV, page 129.

In tests made on transverse and longitudinal wheat sections, clear differentiation in protein content was obtained in samples of both hard and soft wheats; mapping was more pronounced in hard wheat. The stained transverse section of wheat (Fig. 1) showed great differences in protein concentration between outer and inner endosperm, but no zone differentiation as postulated by Cobb. No demarcation line existed between zones of protein-rich and protein-poor endosperm tissues; thus the over-all picture is that of a gradual change in protein content. Figure 1 is representative of the wheat examined. In practically all cases the more deeply stained areas of the wheat endosperm corresponded to "glassy" areas of the wheat kernel.

The more detailed picture of a longitudinal section taken under low magnification (Fig. 2) shows the low level of protein in the pericarp, the high dye adsorption of the germ, and a distinctly high protein content in the aleurone layer.

The results confirm previous findings regarding protein distribution

in the wheat kernel and data on wheat hardness in various layers recently reported (11). Hinton (9) found the following concentration of protein in dissected wheat; pericarp, 4.4%; aleurone layer, 19.7%; endosperm, 13.7%, 8.8%, and 6.2% respectively (from outer to inner endosperm); embryo, 33.3%; and scutellum, 26.7%. The dye-binding capacity of Xylidine Ponceau is correlated with these protein values for various tissues.

The dye-staining technique cannot replace chemical tests, as it supplies only information of a qualitative nature. Though acidic dyes give more consistent and intense staining when applied in buffered form (low pH solutions), solutions employed were unbuffered, because a buffer might affect the dye uptake in various parts of the tested tissue (12).

Despite limitations of the test, it fulfills the prerequisite of a histological procedure (7): The microtome sections are prepared so that no significant alteration takes place in the position of the constituents being investigated; the reagent is fairly specific for the tissue constituent; there is little diffusion of the constituent tested or of the reaction product; and the reaction product is capable of being visualized.

The simplicity and rapidity of the test, as well as the results obtained from testing flour, point to the possibility of employing the procedure as a screening aid in air-classification of flour.

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HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

III. Use of Methyl Green in Estimating Flour Extraction Rate[1]

Y. POMERANZ AND J. A. SHELLENBERGER

ABSTRACT

The quantity of bran in flour may be determined by its high adsorption of methyl green. Flour is shaken with a dilute solution of methyl green, and the dye adsorbed is calculated from the absorbance of the centrifuged supernatant solution.

The estimates of bran in flour, as determined by the proposed method, are highly correlated with the ash content or the color grade of the flour. Staining of flour with methyl green has also been shown useful as an aid in detection of insect fragments, especially in high-extraction flours.

The objectives of milling are to separate endosperm from bran and germ and subsequently to reduce endosperm particles to flour. The efficiency of separation can be judged by several empirical, indirect methods based on measuring one constituent that is concentrated to a larger extent in bran or germ than in endosperm. The most widely accepted chemical measures of flour purity are crude fiber content and mineral or ash content. Although the composition of the branny layers permits basing measurements of flour refinement on determinations of other constituents, such as water-soluble vitamins, phosphorus, and pentosan content, the distribution of those constituents in individual bran layers is not uniform. Nor does relative concentration of the constituents in certain bran layers follow a definite pattern (5,6,7,13). The ash determination is the most widely accepted criterion of flour extraction and efficiency of bran separation. The method is simple, can be performed fairly rapidly, is

[1]Manuscript received May 2, 1960. Presented at the 45th annual meeting, Chicago, Illinois, May 1960. Contribution No. 335, Department of Flour and Feed Milling Industries, Kansas State University, Manhattan.

reproducible, and is well suited for routine determinations. Major objections to the ash test are that mineral-matter content of flour may be affected by factors unrelated to flour extraction, such as variations in the mineral content of the wheat. The ash test also is unsatisfactory in flour fortified by calcium carbonate and in high-extraction flour. Ash content does not always reflect correct quantities of bran, because mineral constituents are higher in the aleurone layer than in the fibrous coatings. Hinton (7) found the gradient in ash content from outer to inner layers of the endosperm to vary in different wheats; more ash was present in the aleurone layer than in the inner endosperm; small portions were found in the fibrous pericarp and testa. While ash determinations are justified in highly refined flour, their use as a yardstick of efficient milling of dark flours may exclude the highly nutritious aleurone layer from flour and replace it with indigestible fibrous bran that has a deleterious effect on baking quality. Hence, a simple and rapid method to express flour grade, irrespective of mineral-matter determination (12), is needed.

The Kent-Jones and Martin Flour Color Grader measures light reflected by a flour slurry (9). The instrument uses a filter with maximum transmittance at 530 $m\mu$. The readings reflect the amount of bran present and are correlated with the flour extraction rate. The method has found wide use in England and in Canada, but its use in other countries has been, so far, limited.

Numerous colorimetric methods have been proposed to determine the bran content of flours. Meyer (11) recommended use of dyes (especially naphthylene blue) as an aid in microscopy of wheat and flour. Recently, Larkin *et al.* (10) have developed a test based on color intensity of bran and germ particles stained with crystal violet. Their method was satisfactory with some flours but not others. Deatherage and MacMasters (4) described a method for direct determination of approximately one-third of the bran in flour.

This study was undertaken to investigate the utility of a dye-adsorption technique to determine the amount of bran in flour. The dye used was methyl green, a basic triaminotriphenyl dye (C.I. 684; synonyms: double green, light green), a coal-tar dye, which has been recommended for staining cutinized and suberized cell walls (3). Chamberlain (2) and Johansen (8) recommend a solution of methyl green in water to stain lignified structures.

The relations between the extent of dye adsorption, the ash content, and color grade (as determined with the Kent-Jones and Martin Flour Color Grader) were studied on commercially and experimentally milled flours.

Materials and Methods

Ninety-two samples of flour were obtained from five commercial mills and one experimental Buhler mill. Moisture and ash determinations were made according to the official methods of analyses of the AOAC (1). Ash content was determined by direct weighing. The results are expressed on 14% moisture basis. Grade color determinations, with the Kent-Jones and Martin Color Grader (9), were made according to the procedure outlined by the manufacturer, involving measurement of light reflected from the surface of a paste prepared from 30 g. of flour and 50 ml. of water. The dye used, methyl green,[2] sometimes contains small amounts of methyl violet but it can be purified. It has an adsorption maximum at 6338A. Methyl green is generally considered to stain more intensely in the presence of acetic acid. Preliminary tests confirmed this view, but an acid suspension was not used because of difficulties in obtaining clear centrifugates. Subsequent tests were continued on a water suspension.

Technical difficulties in measuring adsorbed dye made it easier to measure the concentration of dye in the supernatant and to determine the quantity of bound dye by difference than to measure dye adsorption directly.

The effect of a number of variables in analytical procedure was established. These included: concentration of dye, shaking period, particle size of flour, ratios of dye to bran, and dye adsorption of flour containing different amounts of bran.

After a series of experiments, the following general procedure was followed unless otherwise stated: 2 g. of flour were shaken for 1 hour at room temperature in a 125-ml. stoppered Erlenmeyer flask with 20 ml. of 0.01% methyl green solution. The solution was prepared by diluting a 0.1% stock solution; the stock solution remained stable for more than 1 month at room temperature. After 30 minutes' standing, the supernatant was transferred into a 15-ml. tube and centrifuged for about 15 minutes at 2,000 r.p.m. (1,350 × g). Five milliliters of the clear solution were diluted with 10 ml. of water and the optical density measured with a Beckman (model DU) spectrophotometer at a wave length of 6340A. Amount of adsorbed dye was calculated from the results.

Results and Discussion

Variables Affecting Dye Adsorption. Adsorption of methyl green was found to depend on the concentration of the dye, on the shaking

[2] Coleman and Bell Co., Norwood, Ohio.

period, and on the particle size of flour. The effect of dye concentration is summarized in Table I.

These results show that bran adsorbs far more dye than flour does.

Essentially the same results were obtained when various increments of bran were added to flour and the amount of dye was held constant (20 ml. of a 0.01% solution). Results in Table II are based on tests in which two samples of coarse commercial bran, ground to pass a 40-mesh sieve, were added at various levels to 2-g. samples of flour. Amount of dye adsorbed in each case was calculated after deducting the amount adsorbed by the flour alone, assuming that the addition of bran did not affect the dye adsorption of the flour. The

TABLE I

INFLUENCE OF THE RATIO OF DYE TO FLOUR OR BRAN ON DYE ADSORPTION, EXPRESSED AS MG. METHYL GREEN PER GRAM OF FLOUR OR BRAN

Weight of Flour or Bran	Methyl Green Dye									
	Added	Adsorbed	Added	Adsorbed	Added	Adsorbed	Added	Adsorbed	Added	Adsorbed
mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Flour										
100	5.0	2.5	10.0	4.2	20.0	5.0				
200	2.5	1.6	5.0	2.7	10.0	3.4				
500	1.0	0.5	2.0	1.2	4.0	2.2				
1,000			1.0	0.6	2.0	1.2				
1,500			0.7	0.4	1.4	0.8				
2,000	0.25	0.2	0.5	0.4	1.0	0.7				
Bran										
10	50	35	100	44	200	47				
20	25	17	50	36	100	45				
50	10	8	20	18	40	32				
100			10	9.5	20	18	40	33	100	42
150			8	8	16	13	32	24	55	42
200			5	5	10	10	20	19	40	40

TABLE II

DYE ADSORPTION OF BRAN ADDED AT VARIOUS LEVELS TO 2 GRAMS OF FLOUR

Bran Sample Number	Bran Added to 2 g. Flour	Dye Adsorption of Bran
	mg	*mg dye/g bran*
1	20	4.8
	40	4.5
	60	4.4
	100	4.6
2	40	6.0
	60	5.2
	100	5.3

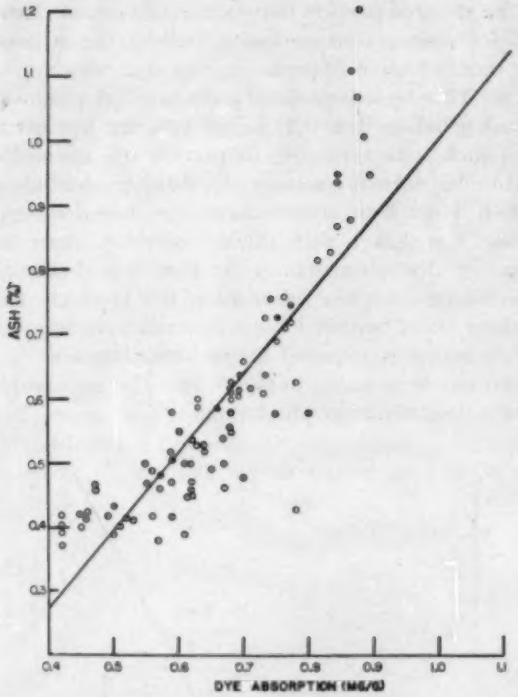


Fig. 1. Scattergram showing relationship between dye adsorption and ash content. ($r = +0.86$)

dye adsorption of the flour used in this series was 0.47 mg. per g.

Dye adsorption of each numbered bran was fairly constant, despite variations from 1 to 5% in the amount added to flour. The ratio of dye adsorption of bran to flour was 10 to 1 for bran No. 1 and almost 12 to 1 for bran No. 2. This ratio essentially did not change by a tenfold increase in added dye. The only advantage of increasing the quantity of dye was that the solutions clarified more easily on centrifugation and did not become turbid even after standing 24 hours. When weaker concentrations of dye were used, the absorbance of the clear solution had to be measured within 4 hours after centrifugation to avoid opalescence. But a more concentrated solution involved additional dilution and difficulties from dye adhering to glassware and stoppers. Stronger dye concentration did not affect relative placing of flours by ash content or grade color; hence, the tests were made with the weaker solution.

To test the effect of particle size on dye adsorption, pure semolina was ground for various time periods to reduce the original product from a size retained on a 20-mesh sieve to that which would pass a 50-mesh sieve. The dye adsorption of the original product increased gradually with grinding from 0.24 mg. to 0.36 mg. dye per g. of product. Though such wide variations in particle size are unlikely to be encountered under normal commercial conditions, particle size affects dye adsorption. Finer flour adsorbs more dye than does coarser flour.

When flour was shaken with the dye solution, there was no significant effect on dye adsorption if the flour was shaken at least 30 minutes. To secure complete adsorption, the products were shaken 1 hour. Shaking times beyond 1 hour sometimes made solutions difficult to clarify and they required longer centrifugation.

Determinations were made in duplicate. The standard error of a dye adsorption determination was 0.011.

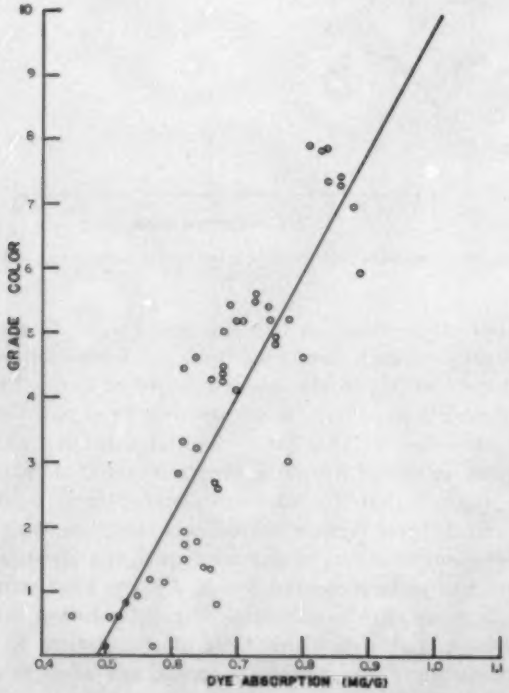


Fig. 2. Scattergram showing relationship between dye adsorption and color grade. ($r = +0.88$)

Relations between Dye Adsorption, Ash Content, and Color Grade. The results on the main series of samples are shown in Figs. 1, 2, and 3.

The correlation coefficients for the above samples were: ash vs. grade color, r = +0.87; ash vs. dye adsorption, r = +0.86; grade color vs. dye adsorption, r = +0.88. All the correlations were significant at the 0.1% point.

The regression equations computed were: A = 1.249C − 0.227 (for calculating ash from dye adsorption); B = 18.985C − 9.193 (for calculating grade color from dye adsorption), and B = 11.630A − 3.160 (for calculating grade color from ash). In the above equations, A = ash content (%), B = grade color, and C = dye adsorption (mg. per g. of material).

Assuming that a flour fairly free from bran contains about 0.35% ash, its dye adsorption would be 0.46 mg. per g. flour, a figure higher than that of ground semolina (0.36 mg. per g.), probably because of the smaller particle size of commercially milled flour.

An increase of 1% ash in flour corresponded to an increase in dye

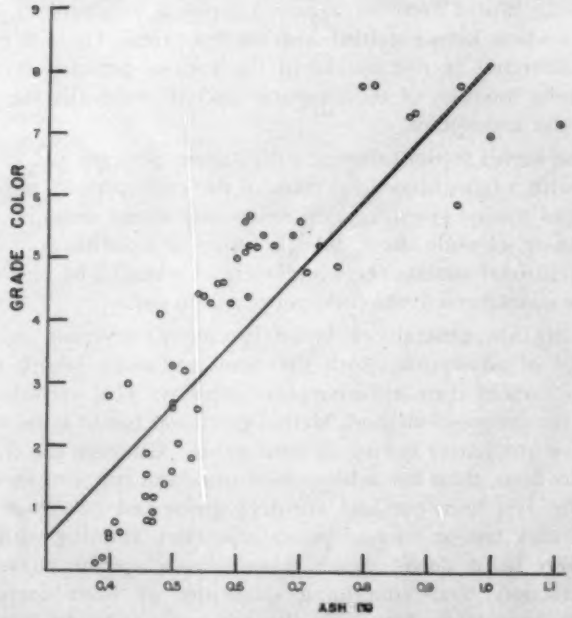


Fig. 3. Scattergram showing relationship between ash content and color grade. (r = +0.87)



Fig. 4. Longitudinal section of wheat kernel stained with methyl green. (10×)

uptake of 0.8 mg. per g. If the mineral matter of bran is 5%, the dye uptake of bran would be 4 mg. per g., which confirms results given in Table II.

Staining Wheat Sections. Figure 4 shows a longitudinal cross-section of a wheat kernel stained with methyl green. There is no measurable difference in dye uptake of the various pericarp layers, and the staining intensity of the aleurone layer is practically the same as that of the endosperm.

Wheat kernel sections showed a deep green pericarp and germ, contrasted with a faint blue-violet color of the endosperm. Use of 0.01% solution of methyl green to stain microtome wheat sections alone, or in a staining schedule after a 0.1% solution of Xylidine Ponceau, provided additional contrast between layers of wheat. The pericarp and germ are stained green; the endosperm, a deep red.

Staining has generally a broad specificity, governed mainly by solubility or adsorption. Both the flour and bran adsorb the dye, but they vary in their dye-adsorption capacity. This variation is the basis of the proposed method. Methyl green was found to be very well suited for qualitative testing of flour grade. Although the dye is adsorbed by flour, flour has a blue-violet tint from traces of methyl violet in the dye; bran particles are deep green and stand out well in either a slick test or microscopic examination. Staining with methyl green also helps detect insect fragments—a special advantage in long-extraction flour containing quantities of bran particles. In stained preparations, bran particles are green, whereas insect fragments are unstained and retain their natural brown color.

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HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS
IV. Mapping the Free Fatty Acids in Germinating Wheat[1]

Y. POMERANZ AND J. A. SHELLENBERGER

ABSTRACT

Changes in free fatty acid content of various tissues of wheat germinated for 8 days were followed by a histochemical method using the base of acridine orange in xylol. Using various substrates showed the staining method specific for fatty acids. Halved kernels stained and viewed under ultraviolet radiation indicated the scutellum as the main site of lipase activity. As the germination period increased, zones farther from the scutellum tissue gradually increased in fatty acids until, after about 8 days, measurable quantities of free fatty acids were present throughout the kernel.

Compared with oil-bearing seeds, particularly castor beans, sound wheat is a poor source of lipolytic enzymes. However, when moisture content is raised, especially on germination, wheat seedlings mobilize reserve storage fat for the developing embryo, and lipase activity increases (18). The lipase activity is located in the outer layers of wheat kernels. Pett (17), using dissected wheat kernels at various stages during germination for 48 hours, studied changes that occur in esterase activity in various tissues. Highest esterase content was associated with the scutellum, and germ lipase activity was considerably less than the scutellum lipase activity. Esterase content of the endosperm was fairly high, but much lower than in the scutellum. Bran contained a significant amount of esterase. Sullivan and Howe (21) found that both clear and low-grade flours had greater lipase activity than patent flours, germ, or bran. Extensively investigated lipids of wheat (14) are concentrated primarily in the embryo and aleurone layer and consist primarily of mixed monosaturated-di-unsaturated glycerides and tri-unsaturated glycerides.

Numerous investigators who have studied changes in fats and fatty acids during germination conclude that the oils disappear fairly rapidly during the course of germination (13,15). Several have reported an increase in fatty acids as germination of seeds progresses (1,2,5,12,16). However, a recent review (20) cites evidence that free fatty acids do not accumulate during the rapid consumption of fat in citrullus cotyledons grown in both light and darkness (3,9).

Methods for measuring lipase activity are based mainly on titration of fatty acids, hydrolyzed from a specified oil substrate under

[1] Manuscript received June 17, 1960. Contribution No. 339, Department of Flour and Feed Milling Industries, Kansas State University Agricultural Experiment Station, Manhattan.

given conditions of time, temperature, and pH. Luchsinger *et al.* (11) developed a sensitive assay procedure based on the glycerol released from a mono-olein emulsion. Ferrigan and Geddes (4) used the procedure to study lipase activity of mill streams from hard red spring wheat. Cereal lipase normally is studied using an artificial substrate sometimes completely unrelated to the oil in the plant tested. The ideal substrate for lipolytic enzyme studies of wheat would be wheat fat (21). With regard to histological staining methods, proof of lipase activity generally is based on an adaptation of Gomori's technique to demonstrate and localize phosphatases in tissue section (8). Glick and Fisher (7) attempted to localize lipase in wheat grain using Gomori's histochemical method and Tween 40 as a substrate. Their failure to find enzyme action in any wheat sections was attributed to low lipase activity of wheat and possible low attack on the ester employed.

The present authors used a saturated solution of the base of acridine orange in xylol to map lipase activity in germinating wheat while it acts on the normally present oil substrate of the wheat kernel. Though no substitute for quantitative analysis, this method shows sites of lipase activity loci and permits tracing transformation of liquids within germinating seed tissues.

Experimental Work

Materials. Selected, sound kernels of hard red winter wheat were treated 2 minutes with a 1:1000 mercuric chloride solution to retard mold growth, rinsed with distilled water, and placed (on consecutive days) between two layers of moist filter paper in a covered Petri dish. The samples were germinated at room temperature for 8 days and then removed for testing.

Acridine orange (AO) is a basic dye (C.I. No. 788)[2]. The staining dye base was obtained by precipitating a 0.2% water solution of the filtered dye, by dropwise addition of *N* sodium hydroxide solution and an excess of 1 to 2 drops of the alkali. The precipitate was washed with water (pH 8.0) and, after vacuum-drying in a desiccator, kept in a stoppered glass bottle. The precipitated dye base remained stable for months. A saturated solution of the dye base in xylol was prepared for use daily by shaking 15 minutes and filtering. The lipase used was soluble wheat germ lipase from Mann Research Laboratories.

Methods. Pictures were taken using UV radiation with a predomi-

[2] Coleman and Bell Company, Norwood, Ohio.

nant wave length of 3,660 A, and high-speed Ektachrome film. As it was desirable to obtain a background of the unstained wheat tissues, no effort was made to eliminate the small amount of visible light from the radiating UV source.

Results and Discussion

Use of the base of AO in staining lipid materials is based on findings by Knaysi (10). The free base of a number of dyes was reported to have colors different from their respective salts or soaps. The dye base is soluble in xylol but practically insoluble in water. Dyes recommended by Knaysi showed poor contrast, so a dye more suited to histological work was sought. AO dye shows, in addition to the above properties, a very pronounced polychromatic fluorescence, which gives a multiple color effect that depends on the concentration of dye bound by various tissues. The color changes from green through yellow-orange to red with increasing concentration.

Specificity. Several triglycerides, fatty acids, pyruvic acid, and lactic acid, were used to test the specificity of the staining reaction. A small drop or small piece of solid material was placed in the cavity of a spot plate and covered by adding 0.5 ml. of a saturated dye solution in xylol; the color was observed after 3 minutes both under

TABLE I

COLORATION OF VARIOUS SUBSTRATES WITH ACRIDINE ORANGE BASE, USING ULTRAVIOLET RADIATION

	SUBSTRATE	COLOR IN UV
0	Control	Green
1	Tristearin	Green
2	Tripalmitin	Green
3	Refined commercial corn oil	Green
4	Tween 80	Green
5	Crude wheat germ oil[a]	Yellow-orange
6	Crude corn oil[a]	Yellow-orange
7	Commercial soybean lecithin	Orange-reddish
8	Glycerine	Green
9	Tween 80 incubated for 2 hours with lipase	Small red droplets
10	Butyric acid	Red-orange
11	Lauric acid	Red-orange
12	Linoleic acid	Red-orange
13	Myristic acid	Red-orange
14	Octanoic acid	Red-orange
15	Oleic acid	Red-orange
16	Palmitic acid	Red-orange
17	Stearic acid	Red-orange
18	Undecylic acid	Red-orange
19	Lactic acid	Yellow-orange
20	Pyruvic acid	Yellow-orange

[a] Diethyl ether-soluble material, freshly prepared in the laboratory from wheat germ and ground corn, respectively.

daylight and under UV radiation. Results obtained with both sources were identical except that the UV gave better contrast; those reported in Table I were obtained using this source.

Results summarized in Table I show that the staining is highly specific in differentiating between lipids and fatty acids derived from lipid material. However, any acidic substance soluble in xylol caused a shift in color of the AO base solution; this was observed on addition of lactic or pyruvic acid. These intermediary metabolites are likely to be found in germinating wheat but not necessarily a result of storage-fat breakdown. This should constitute only a minor limitation to the use of the method. The solubility of pyruvic and lactic acid in xylol is low, especially compared with their practically infinite solubility in solvents normally employed (i.e., 50% alcohol) in acidimetric determinations of lipase activity.

Distribution of Fatty Acids in the Wheat Kernel. Major sites of high lipid concentration, and generally free fatty acids in a dormant sound wheat kernel, are the germ and the aleurone. (See part I of this series, Figs. 1 and 2; p. 129.)

Wheat sections, prepared with a freezing microtome from totally untreated material, were stained by covering with the dye solution in the cavity of a spot plate. After 5 minutes, the sections were blotted on filter paper and allowed to air-dry. On observation under UV radiation, the germ and aleurone showed a brilliant red fluorescence in contrast to the small dye uptake resulting from the low concentrations of fatty acids of the endosperm. Compared with controls, stained diethyl ether or xylol-extracted wheat sections showed little coloration of the germ or aleurone layer. Such practically fat-free sections were subsequently covered with a thin layer of crude wheat germ oil, prior to staining. After 3 hours, to allow the wheat to adsorb oil, the oil-covered sections showed a brilliant fluorescence on staining.

Finally, wheat kernels, kept overnight between moist filter paper, were divided into three lots. The distal ends of the kernels of one lot were injected, by means of a syringe, with 0.01 ml. of distilled water. Kernels of a second lot received an equivalent volume of a 0.1% suspension of lipase; and the third lot was treated with a heat-inactivated suspension of lipase preparation. The kernels were kept between moistened filter paper at room temperature 18 hours, then halved and air-dried 1 hour. The air-dried halves were stained and observed. After staining with AO, the wheat kernels to which active lipase was added were more deeply colored than the controls. The difference in color intensity was far less than that observed between

sound kernels and kernels germinated for longer periods (about 8 days), indicating that the distribution of lipase is not the only factor governing the presence of fatty acids in various tissues.

Results of these tests indicate that the staining method is well suited for following the location of fatty acids produced by lipase activity in germinating wheat.

Figures 1 through 6 appear at the end of this article.

Before discussing the results of changes in the interior of the wheat kernel, it is pertinent to consider Figs. 1 and 2 which show the dorsal side of wheat halves, with emerging shoots. The staining technique permits one to observe the presence of the thin cutin lamellae which are known to be formed on the outer walls of the epidermal cells of leaves and stems. Cutin (19) is a complex mixture consisting mainly of unsaturated fatty acids that have undergone condensation and oxidation. The relatively high concentration of fatty acids in the thin varnishlike skin on the surface governs the dye uptake and brilliant coloration.

Changes during Germination. Figures 3 to 5 show clearly that the scutellum is the major site of lipase activity in the germinating wheat. Activity in the aleurone layer remained fairly constant. Even at advanced stages of germination (Fig. 4), parts of the wheat endosperm near the aleurone but distant from the scutellum were low in free fatty acids.

Figure 6 shows the pattern of fatty acid distribution in germinating maize.

The results show that during germination the enzymes elaborated by the scutellum eventually reach the more distant parts of the kernel until, at the final stages of germination, all lipid material may be converted to free fatty acids which become available to the developing seedling. Though the concentration of the endosperm fat is low, the quantity of fat present is about one-third of that in the whole kernel. It is possible that during the later stages of germination the reserve fat of the endosperm is used, along with the more concentrated sources of lipid material in the germ and aleurone.

Although the results obtained show that progressive germination is concomitant with the presence of free fatty acids in new "zones" more distant from the scutellar site of major lipase activity, it is impossible to account for the source of these fatty acids on the basis of the staining technique employed. The results of Pett (17) point to a redistribution of lipases on germination of wheat, resulting in an abrupt fall of the lipase activity in the scutellum along with a small increase in other tissues. Malhotra (13) has shown that in

germinating maize, fat decreased in the seed from 4.9 to 4.1% during 8 days of germination. When the endosperm and embryos of maize were separated at the different stages of germination before being analyzed, fat increased in the endosperm with a corresponding decrease in the embryo.

The amount of free fatty acids present in different parts of a germinating wheat kernel therefore may be governed, in addition to conditions of growth, by a number of additional factors such as: (a) breakdown of lipids due to the secretion of lipases from the scutellum or activation of lipases at new sites of the endosperm, which would bring about a net increase in fatty acids; (b) redistribution of lipids, but not changing total fatty material content in the whole kernel.

The answer to these questions cannot be obtained by the staining method employed, but the method can supplement information obtained by chemical methods. It is simple and shows high specificity. It is promising as it permits staining fatty acids specifically and not fats or oils as such.

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Fig. 1, page 107. Cross-section of kernel (50×), showing coloration of the aleurone viewed by reflected ultraviolet light.



Fig. 2, page 107. Longitudinal section through both cheeks, crease, and part of germ (20×), showing coloration of aleurone and germ viewed by reflected ultraviolet light.



Fig. 3, page 107. Ground wheat particles (20×), showing coloration of particles originating from aleurone or germ tissues.



Fig. 4, page 107. Flour (50×), showing coloration of particles originating from aleurone or germ tissues.



Fig. 1, page 111. Stained transverse sections of wheat, showing differences in protein content between outer and inner endosperm. Microscope not used, but magnification of approximately three times normal size obtained by use of extension tubes on camera.



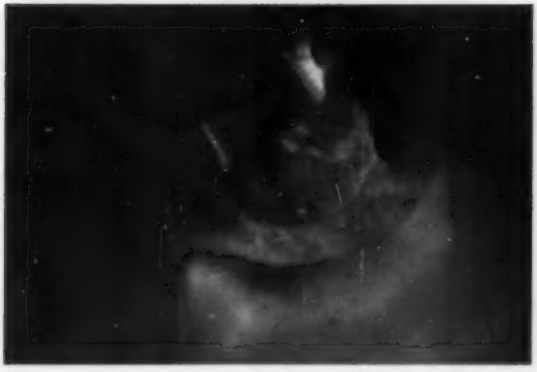
Fig. 2, page 111. Stained longitudinal section of germ and adjacent layers of wheat kernel showing areas of high protein concentration in the embryo and aleurone layer and the low protein content of the pericarp. (20×)



Figs. 1 and 2, page 126. Dorsal side of longitudinal wheat halves after 1 and 2 days of germination, respectively, viewed by reflected light. (20×)



Figure 2



Figs. 3 and 5 inclusive, page 126. Halves of germinating wheat, longitudinal, after 2, 4, and 8 days of germination, respectively. In Fig. 5 the shoot is seen along the half of the kernel. (20×)



Figure 4



Figure 5



Fig. 6, page 126. Halves of germinating corn, longitudinal, taken with ordinary camera, employing extension tubes; viewed with reflected ultraviolet light. Upper row, exposed interior; lower row, dorsal side showing emerging shoots. From left to right: after 1, 3, 6, and 8 days of germination.

HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

V. Sulfhydryl Groups: Their Localization in the Wheat Kernel[1]

Y. POMERANZ AND J. A. SHELLENBERGER

ABSTRACT

The method of Barrnett and Seligman for histochemical visualization of protein-bound –SH groups in animal tissue cells has been applied to localize sulfhydryl groups in the wheat kernel. Wheat sections, fixed in 2% trichloroacetic acid in ethanol, were reacted with a buffered solution of 2,2′-dihydroxy-6,6′-dinaphthyl disulfide. The colorless, insoluble oxidation product was monoazotized to form a colored violet dye at sites of protein-bound sulfhydryls. Major sites of –SH groups were the aleurone layer and the germ. The histochemical method was specific and inhibited by oxidizing or –SH-blocking reagents.

Despite the importance of the sulfhydryl and the disulfide groups in flour proteins, their determination has been difficult. Nearly all methods for the estimation of sulfhydryl groups of proteins (1,3) are nonspecific, because functional groups other than sulfhydryl react with the reagents.

Barrnett and Seligman (2) have used a number of histochemical methods to demonstrate sulfhydryl groups. To improve the sensitivity and specificity of sulfhydryl histochemistry, they developed a new reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD). The reagent contains a disulfide linkage, a specific oxidizing group, and a naphthol moiety for coupling, to form an azo dye. The basis for using this compound depends on specific oxidation of sulfhydryls at an alkaline pH. The reaction involves, in addition to reduction oxidation, transfer of the chromogenic naphthol moiety to protein and formation of an oxidation product, from which a colored compound is produced. Barrnett and Seligman suggested producing an azo dye by coupling with tetra-azotized diorthoanisidine. Other monocoupling diazonium salts have been proposed by Cafruny *et al.* (4) and by Teiger *et al.* (7).

The reaction between DDD and protein-bound –SH groups is specific (1). The staining reaction in animal tissues was inhibited by oxidizing the –SH groups with dilute iodine and by using blocking agents such as N-ethylmaleimide and iodoacetate.

Hyde and Paliwal (6) recently tested the applicability of the Barrnett-Seligman (2) method to plant material. N-ethylmaleimide

[1]Manuscript received September 1, 1960. Contribution No. 347, Department of Flour and Feed Milling Industries, Kansas State University, Manhattan.

was found to be a reliable thiol-blocking reagent. The DDD reaction also could be partly blocked by 0.1*M* *p*-chloromercuribenzoate, but the blockage was partly overcome if the DDD reaction was continued a long time. The reaction was blocked with neither iodine nor iodoacetate.

Sulfhydryl groups are known to be important in animal tissues (1,5) and in plant tissues (6).

The purpose of this investigation was to demonstrate histochemically the presence and distribution of sulfhydryl groups in the wheat kernel and, if possible, to use the information obtained from histochemical studies to determine sulfhydryl groups in wheat flour. Localizing the sulfhydryl groups histochemically in the wheat kernel also provides the possibility of revealing particles originating from sulfhydryl-rich tissues, in wheat flour.

Materials and Methods

Materials. Selected, sound kernels of hard red winter wheat or of dent corn were cut at the distal end and kept overnight in 2% trichloroacetic acid (TCA) in 80% ethanol solution. The kernels were cut into transverse sections about 20μ thick for microscopic observation with transmitted light, and into sections 50μ thick for observation were reflected light. Materials included were:

Wheat germ, wheat bran, and wheat flour fixed overnight in 2% TCA in 80% ethanol
2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD)[2]
Tetra-azotized diorthoanisidine[3]
Fast Blue RR[4]
0.1% aqueous solutions of potassium bromate, potassium iodate, potassium iodosobenzoate, and N-ethylmaleimide[5]
Saturated water solution of potassium periodate
0.1*M* Michaelis barbital buffer, pH 8.5
Dilute acetic acid, pH 4.0–4.5
0.067*M* Sörensen phosphate buffer, pH 7.4
Ethanol: 95, 70, 50, and 10% (water) solutions
Diethyl ether

Methods. When used in excess at pH 8.5, DDD (I, Fig. 1) forms, with active sulfhydryl groups of fixed tissue proteins, a colorless substance (II) which can be converted to an intensely colored azo dye (IV) by a coupling reaction. The colorless oxidation product (II) is insoluble in water and diethyl ether, so that an excess of DDD and the reaction by-product (III) can be washed from the tissues with organic solvents. If coupling is carried out with tetra-azotized diorthoanisidine, either a red color (due to monocoupling) or a blue color (due to di-

[2] From Schwartz Laboratories, Mount Vernon, N. Y.
[3] From Schwartz Laboratories, Mount Vernon, N. Y.
[4,5] From Dajac Laboratories, Philadelphia, Pa.

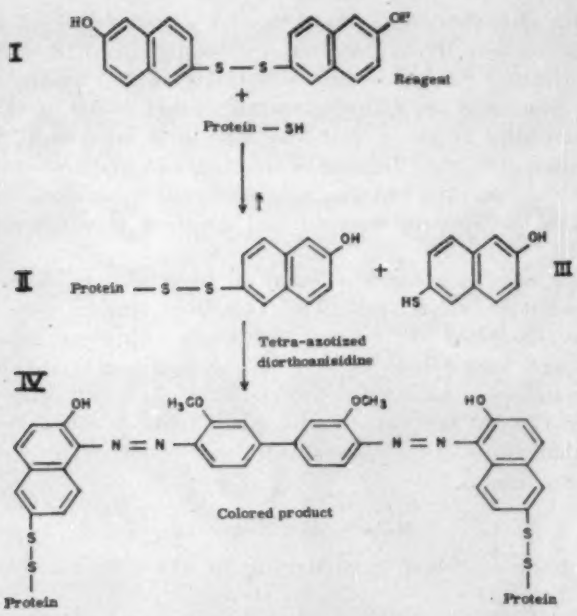


Fig. 1. Scheme of the Barrnett-Seligman histochemical procedure for determination of thiol groups. (Science 116: 323–327; 1952. Reprinted by permission.)

coupling) develops at the sites of protein sulfhydryl groups (IV). The mechanism of the staining procedure is outlined in Fig. 1.

Preliminary experiments were made with tetra-azotized dianisidine. Though results were satisfactory, the reagent was replaced with the Fast Blue RR monocoupler recommended by Cafruny *et al.* (4). This was done to obtain a single-colored (violet-red) product of monocoupling, as it was desired to use the information obtained from histochemical tests for the quantitative determination of –SH groups in flour.

Wheat sections were mounted on slides with minimal albumin for the staining procedure. The albumin did not interfere with the histochemical reaction. The slides were incubated 1 hour at 50°C. in a filtered alcohol buffer mixture (prepared by mixing 35 ml. 0.1*M* Michaelis buffer and 15 ml. 95% ethanol) which contained 25 mg. 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD). After cooling 10 minutes at room temperature, the slides were rinsed briefly in water and washed 10 minutes in dilute acetic acid. The excess reagent and reaction by-product were extracted with two changes of ether for

5 minutes, after slides were passed through a graded series of alcohols. The sections were rehydrated and, after being rinsed in water, were stained exactly 2 minutes at room temperature with a freshly prepared filtered solution of the coupling reagent (either 50 mg. tetra-azotized diorthoanisidine or 50 mg. Fast Blue RR) in 50 ml. 0.067*M* Sörensen phosphate buffer. Both the time of coupling and freshness of the solution affect the staining and they must be rigidly controlled. The slides were washed in running tapwater and the cover glass mounted with glycerol.

Specificity of reaction was tested on fixed sections, wheat germ, bran, and flour, respectively. The TCA-fixed samples were washed with two changes of 80% ethanol and briefly with water. Subsequently, they were kept 1 hour at 30°C. in the oxidizing or –SH-blocking solution and, prior to reaction with DDD, washed with three changes of water. Control samples were passed through the same procedure, except that distilled water was used instead of the oxidizing or –SH-blocking solutions.

Results and Discussion

The results obtained from staining of wheat sections are shown in Figs. 2, 3, and 4.

In Fig. 2, the whole transverse section is shown. In Figs. 3 and 4, parts of the section shown in Fig. 2 are given at higher magnification. These results show clearly that the major sites of sulfhydryl groups in the wheat kernel are the germ and the aleurone layer. The sulfhydryl content of the endosperm is much less than that of the aleurone layer or the germ. In the endosperm, there is a gradual decrease of

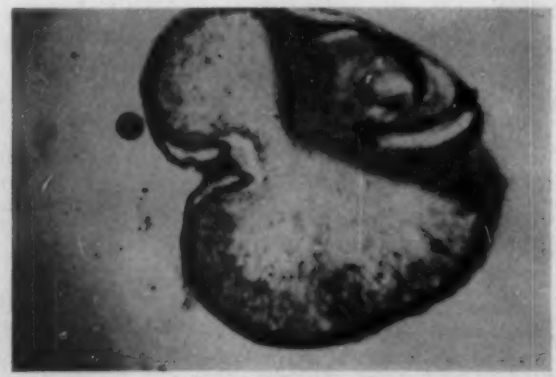


Fig. 2. Transection through whole-wheat kernel near the germ end (20×).

sulfhydryl groups from the outer layers to the central ones; the smallest concentration of –SH groups is in the central portions of the endosperm and the cheeks.

Figure 5 shows in more detail the distribution of sulfhydryl groups in the pericarp, seed coat, and adjacent layers, as well as in the aleurone layer.

Figure 6 shows that the pattern of distribution of sulfhydryl groups in the corn kernel is the same as in wheat; the major sites of sulfhydryl groups are the aleurone layer and the germ.

Data on the specificity of the reaction are given in Table I.

The reaction is inhibited to a large extent, or completely, by the



Fig. 3. Transection through wheat kernel; shows details of pericarp and adjacent tissues (50×). Detail of Fig. 2.



Fig. 4. Transection through wheat kernel shows details of embryo and layers adjacent to crease (50×). Detail of Fig. 2.



Fig. 5. Transection through pericarp and adjacent tissues in wheat (200×).

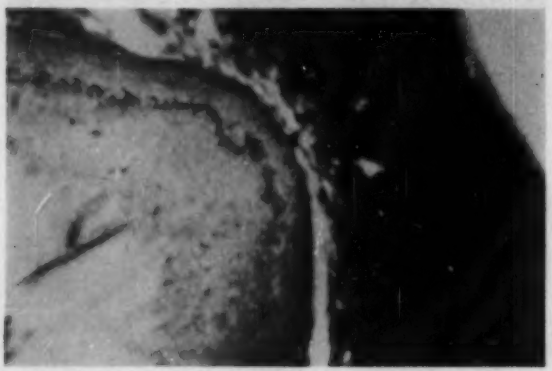


Fig. 6. Transection through pericarp and adjacent tissues, endosperm, and embryo of a corn kernel (20×).

oxidizing or blocking reagents employed. Action of the various agents used differs widely. Potassium iodosobenzoate was most effective; N-ethylmaleimide was a close second. Iodate and periodate caused high inhibition of the reaction, whereas bromate (extensively used as a flour-oxidizing agent) was least effective although it decreased staining intensity slightly.

Though the Barrnett-Seligman procedure is generally accepted as specific for –SH groups, no blocking reaction completely prevented the DDD reaction (6). During the reaction, a slightly alkaline medium (buffered at pH 8.5) was employed. It made the –SH groups more reactive (but at the same time it opened S–S linkages). Generally, animal or plant tissues that give positive tests for sulfhydryls also contain disulfides. This is because sulfhydryls and disulfides provide

TABLE I

EFFECT OF OXIDIZING AND BLOCKING REAGENTS ON THE HISTOCHEMICAL TEST OF SULFHYDRYL GROUPS IN WHEAT PRODUCTS

TREATMENT	COLOR OF TESTED SUBSTRATE			
	Sections	Germ	Bran	Flour
Control	Germ and aleurone very deep violet; endosperm violet	Very deep violet	Very deep violet	Violet
Bromate	Germ and aleurone deep violet	Deep violet	Violet	Violet
Iodate	Slight violet	Very slight violet	Very slight violet	Unstained
Periodate	Violet	Very slight violet	Very slight violet	Unstained
Iodosobenzoate	Unstained	Unstained	Unstained	Unstained
N-ethylmalemide	Very slight violet	Very slight violet	Unstained	Unstained

a truly reversible intracellular oxidation-reduction system. This may be of special importance in the histochemistry of wheat products where the predominant and relatively abundant disulfide links are liable to be broken to an appreciable extent by the DDD reagent and thus can interfere with the complete color inhibition. Even if a predominance of either sulfhydryls or disulfides is formed in certain tissues, resulting from a shift in equilibrium of the reversible redox system, prolonged incubation in DDD may affect the staining reaction or partially overcome the blockage in tests employing blocking agents.

The results of the present investigation show that the presence in wheat flour of particles originating from the aleurone and germ tissues accounts for the high levels of thiol groups in low-grade milled wheat products. The relation of sulfhydryl content to bromate requirements and the possible application of the histochemical test for visualization of protein-bound –SH groups to the quantitative estimation of sulfhydryls in wheat flour are being investigated.

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THE FORMATION OF DOUGH AND BREAD STRUCTURES[1]

I. The Ability of Starch to Form Structures, and the Improving Effect of Glyceryl Monostearate

G. Jongh

ABSTRACT

Dough prepared from starch instead of flour behaves like a concentrated stable suspension; it shows, among other properties, dilatancy.

Such a dough acquires plastic properties when small quantities of glyceryl monostearate (GMS) are added (e.g. 0.1%).

Starch bread without the additive has a stiff crumb with an irregular, very coarse structure. By the addition of 0.1% GMS a loose crumb with a fine and regular structure is obtained.

The influence of GMS on the texture of the dough is explained by assuming that GMS is adsorbed on the surface of the starch granules, and that, consequently, the stable system is transformed into a flocculated one. In accordance with this, GMS greatly increases the rate of settling and the sediment volume in a 2% starch suspension.

The large decrease of the rigidity of the crumb by GMS is probably caused by weakening of the bindings between the swollen starch granules.

It is generally accepted that the gluten gel, formed in mixing, determines in the main the properties of bread dough[2] (1,12). The starch granules mixed with this gluten gel are usually considered unimportant for the rheological properties of the dough and for the gas retention of the dough. According to the most accepted view, the gluten acquires greater firmness during the baking process and finally forms the network to which the crumb owes its coherence.

Rotsch (8,9,10) made bread with a good crumb from dough in which wheat protein was replaced by other gel-forming substances. He was not, however, successful in finding a suitable replacement for starch. Rotsch concluded from his tests that the bread crumb owes

[1] Manuscript received March 29, 1960. Contribution from the Institute for Cereals, Flour and Bread T.N.O. at Wageningen, The Netherlands.

[2] Bloksma, A. H., and Hlynka, I. Basic considerations of dough properties. *In* I. Hlynka, ed.: "The physical basis of wheat quality." AACC Monographs, Vol. III; in preparation.

its coherence to partly gelatinized starch only. He attributed differences in the crumb properties to varying degrees of gelatinization of the starch. In accordance with the current opinion he assumed, however, that in the dough the carbon dioxide is retained by the gluten gel. Therefore he also supposed that doughs without gluten can only retain the gas if another gel takes over this role from the gluten. So Rotsch explained the fact that there is no substitute for starch on the basis only of the role of starch during and after baking.

Hess (3) made the supposition that the properties of normal dough are determined not only by gluten, but also by electrostatic binding between starch and gluten. According to Hess, both gluten and starch form a coherent network in the bread crumb.

With the aim of providing a basis for studying the formation of structures in normal dough and bread, baking tests were made with separate components of flour. The present experiments were started with a system which, apart from sodium chloride and the necessary ingredients for fermentation, contained only starch and water in about the same proportion as normal dough. Tests were made to determine if a structure resembling that of normal bread could be developed in such a system by mixing, fermentation, and baking.

The formation of more or less permanent structures can only be expected in systems in which the particles adhere one to another. In suspensions of ungelatinized starch there are, however, repulsive forces between the starch granules (2,5). Therefore an attempt was made to cause flocculation of starch in the system by adding small quantities of a surface-active agent. In this connection the effect of glyceryl monostearate on the development of structures in dough and bread was studied.

Materials and Methods

Materials: Starch. Commercial unmodified thick-boiling wheat starch was used, containing 0.54% fatty acids, 0.15% glycerides of fatty acids, 0.19% protein (N × 5.7) and 0.22% ash (14% m.b.). For the determination of fatty acids and the glycerides of fatty acids, the following procedure was used: the flour was boiled with diluted hydrochloric acid, then ethanol was added and the solution was extracted with petroleum ether. An aliquot sample of the petroleum ether layer was evaporated to dryness, and the free fatty acids and the glycerides were determined by titration with 0.1*N* isobutyl alcoholic potassium hydroxide before and after saponification respectively.

Glyceryl monostearate (GMS). A trade preparation of the self-emulsifying type was used, containing about 50% pure glyceryl

monostearate, 40% distearates, practically no triglycerides, and 3% free glycerin. It had an iodine value <4 and a saponification equivalent of 160. Where percentages of GMS are mentioned hereafter they refer to this trade preparation. It was added as an emulsion in water (1:9).

Tests on the Stability of Dilute Starch Suspensions. Suspensions were made from 2 g. wheat starch in distilled water with various additions of GMS. The volume of the suspension was made up to 100 ml. in a stoppered calibrated cylinder. After shaking, the volume was read at regular intervals.

Baking Tests. Starch doughs were prepared from 300 g. wheat starch (14% m.b.), 180 g. water, 6 g. sodium chloride, 12 g. sucrose, and 18 g. yeast (basic formula). To this various amounts of GMS were added.

The dough was mixed for 5 minutes in the large bowl of the farinograph, after which a portion of dough (250 g.) was directly transferred into a pan and fermented for 15 minutes. After this it was stirred, proofed for 45 minutes at 30°C., and finally baked for 20 minutes at 240°C.

Farinograph. A two-speed farinograph was used. Generally the normal speed (velocity of the blades 58 and 87 r.p.m.) was used in this study. (Some farinographs have, however, been obtained at half speed.)

Microscopic Examination of the Bread Crumb. Sections 15μ thick were cut by means of a microtome from frozen bread crumb. The sections were embedded in glycerol and studied under the microscope.

Results

Flocculation of a Dilute Starch Suspension. In a starch suspension the granules do not attract each other (2). Such systems are called stable. The stability holds even if large quantities of electrolytes are present. For instance, the presence of 3 g. sodium chloride in 100 ml. 2% starch suspension did not cause flocculation. The stability was demonstrated by the fact that settling is slow for a suspension of particles with the size of starch granules and also by the small volume of sediment. In the presence of GMS, however, large aggregates were formed, resulting in a large sedimentation volume.

Table I shows the course of sedimentation in 2% starch suspensions with and without added GMS respectively. In the experiment mentioned the pH of the GMS solution (originally 8.4) was previously brought to the pH of the starch suspension (5.7) with hydrochloric

TABLE I

SEDIMENTATION VOLUMES OF 2% STARCH SUSPENSIONS

FLOCCULATION AGENT	TIME AFTER SHAKING (Minutes)					
	3	5	10	15	30	120
g/100 ml	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*
	0.3	0.5	1.0	1.4	2.5	3.7
GMS, 0.1 g.	22.0	31.0	23.8	21.4	18.9	17.9

acid. Flocculation occurred, however, in about the same measure if the pH of the GMS solution was left unchanged.

Properties of Starch Doughs with Various GMS Additions. The resistance during mixing of starch dough is naturally dependent on the amount of water. As long as this was too small for complete moistening of the starch, the curve registered by the farinograph was very irregular. When there was sufficient water, however, the consistency recorded by the farinograph remained constant with continuous mixing after about 5 minutes. The necessary minimum amount of water for this varies. At least 54.8% water had to be added to the commercial starch (14.0% m.b.) used in the tests presented here; with this water content the maximum consistency was obtained, which (5 minutes after the start of mixing) amounted to 400 B.u. In the baking tests described, 60% water was added to the commercial starch (14% m.b.). Then the consistency of a dough containing no ingredients but starch, water, sodium chloride, yeast, and sugar, was 290 B.u. Baking tests were also carried out with doughs to which GMS had been added. Table II shows that the addition of GMS, even in small quantities, had a big influence on the consistency of the dough.

Figure 1 shows farinograms of a starch-water mixture without GMS and farinograms with 1% GMS. Farinogram A was obtained by mixing 300 g. of starch (14% moisture) and 176 g. of water at

TABLE II

CONSISTENCY OF STARCH DOUGHS MEASURED IN THE FARINOGRAPH 5 MINUTES AFTER THE START OF MIXING (NORMAL SPEED)

ADDITIVE	AMOUNT ADDED TO BASIC FORMULA[a] (% of Starch, 14% m.b.)		
	0	0.1	1
	B.u.	*B.u.*	*B.u.*
Water	290	290	265
GMS	290	235	185

[a] Basic formula: 300 g. wheat starch (14% m.b.), 180 g. water, 6 g. sodium chloride, 12 g. sucrose, 18 g. yeast.

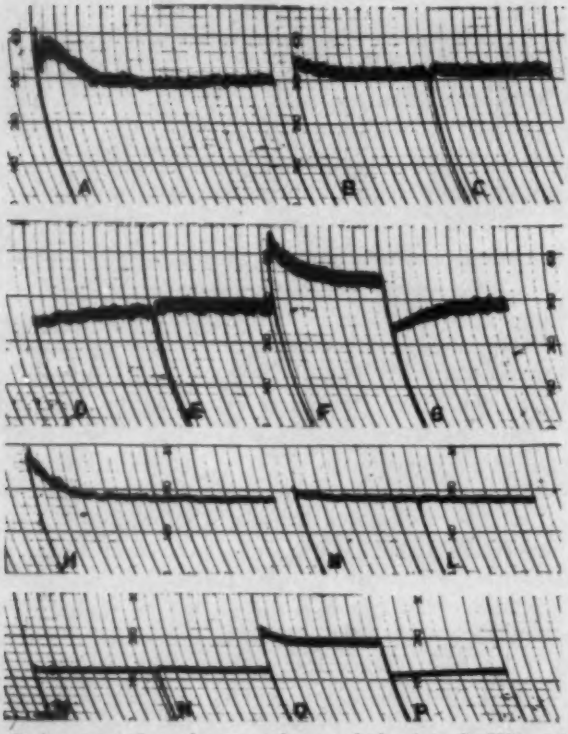


Fig. 1. Farinograms of starch-water mixtures. Series A to G: 300 g. wheat starch (14% m.b.) plus 176 g. water; series H to P: plus 1% GMS. Mixing time, 10 minutes for A and H, 5 minutes for the other curves; mixing speed, 58 r.p.m. for A, B, C, F, H, K, L, and O; 29 r.p.m. for D, E, G, M, N, and P; in the case of B, D, K, and M, mixing was preceded by 5 minutes' dough rest; after the recording of curves B, D, H, and M, mixing was interrupted for 20 seconds; for the rest, mixing proceeded without interruption.

normal speed in the large bowl. Soon after the start of mixing the consistency reached a maximum; it then decreased, and reached a practically constant value after 2.5 minutes. Curves B, C, D, E, F, and G were obtained from the same mixture as curve A. Between A and B mixing was interrupted for 5 minutes. After this rest period a somewhat higher consistency was measured, which again decreased during mixing (curve B). An interruption of mixing, just long enough for the pointer of the farinograph to reach zero on the scale (20 seconds), had practically no influence on the measured consistency (curve C). Curve D was obtained by mixing at the slow speed, after a rest period of 5 minutes. In contrast to the comparable experi-

ment at normal mixing speed (curve B), the consistency now showed an increase during mixing. Just as in the case of fast mixing, the interruption of slow-speed mixing during 20 seconds had no visible effect on the consistency measured (curve E). It was further shown that with mixing at normal speed the same type of curve was obtained when it immediately followed a period of slow mixing (curve F) as when it was preceded by a rest period (B). Likewise the type of curve obtained with slow-speed mixing was the same after a rest period (D) and after a period of normal mixing speed (G).

Curves H, K, L, M, N, O, and P were obtained in the same way as the curves from the first series, the only difference being that 1% GMS had been added. All these curves were significantly lower and had a much smaller band width than the comparable curves from the first series. They still showed the relative differences in shape which have been described for the first series, but in a much lesser degree.

The starch dough of the basic formula had a more fluid character than flour dough: it could easily be poured from one vessel into another. It showed dilatancy, however: when the dough was stirred, the viscosity increased, while the mobility decreased; if the stirring was stopped the dough returned to its former condition.

Even with the addition of small quantities of GMS (e.g. 0.1%) the dough lost dilatancy, while the rate of flow under the influence of gravity decreased. If 0.5% GMS was added, flow was already extremely slow, while by further increasing GMS addition the starch dough acquired more and more the character of a solid paste. At the same time the rate of dough rise during fermentation decreased.

Influence of GMS on the Properties of Starch Bread. The addition of GMS had a great influence on the structure of the starch bread, as may be seen from the cross-sections shown in Fig. 2. Starch bread baked from the basic dough had an irregular and very coarse struc-



Fig. 2. Loaves from starch. Left to right: basic formula, and basic formula with additions of 0.05, 0.1, and 1% GMS (relative to starch with 14% moisture). Basic formula: 300 g. wheat starch (14% m.b.), 180 g. water, 6 g. sodium chloride, 12 g. sucrose, 18 g. yeast. Bread was baked from 250 g. of dough.

ture with thick cell walls. The bread was already hard immediately after cooling. By the addition of 0.05% of the trade preparation of GMS (that is, 0.025% of the pure compound) a regular and reasonably fine crumb was obtained. Larger additions produced a rapid increase in the fineness of the grain and at the same time in the crumbliness of the bread. When 5% of GMS was added the dough scarcely rose, except when at the same time a higher amount of water was used. With 5% GMS and 85% instead of 60% water, bread with a fine though very loose crumb was obtained. The consistency of the dough measured with the farinograph was then almost nil.

The influence of various GMS additions on the moisture retention of the bread crumb during baking is shown through the results of moisture determinations in the crumb of fresh, cool loaves, given in Table III. The addition of 0.1% GMS was accompanied by an increase of moisture content of 2% (relative to starch with 14% moisture). This increase was significant on a $P = 0.01$ level. Further changes in the moisture content, upon the addition of 0.5 and 1% GMS, were not significant. The moisture was, however, significantly higher upon the addition of 5% GMS than at the addition of 0.1% GMS.

TABLE III

INFLUENCE OF VARIOUS GMS ADDITIONS ON THE MOISTURE RETENTION OF THE BREAD CRUMB

AMOUNT OF GMS ADDED TO BASIC FORMULA [a] (% of Starch, 14% m.b.)	MOISTURE CONTENT [b] (% of Bread Crumb)	MOISTURE CONTENT (% of Starch, 14% m.b.)
%	%	%
0.0	32.7	43.1
0.1	34.4	45.1
0.5	35.0	46.6
1.0	34.9	46.6
5.0	35.9	50.6

[a] Basic formula: 300 g. wheat starch (14% m.b.), 180 g. water, 6 g. sodium chloride, 12 g. sucrose, 18 g. yeast.

[b] Average results of three equal series of baking tests.

Microscopic Examination of the Crumb. The micro structure of the cell walls in the crumb of starch bread changed considerably under the influence of GMS. Figure 3 shows that when no GMS had been added, the starch granules in the cell walls of the bread crumb had completely grown together. With 0.02% GMS the contact between starch granules was already broken at large parts of their surfaces. Additions of 0.05 and 0.1% GMS caused a further decrease in the contact between the starch granules. At the same time, the shape of

the granules became less elongated. With 1% GMS the starch granules practically showed the form of loose, somewhat swollen granules. With 5% GMS the coherence between the starch granules in the bread

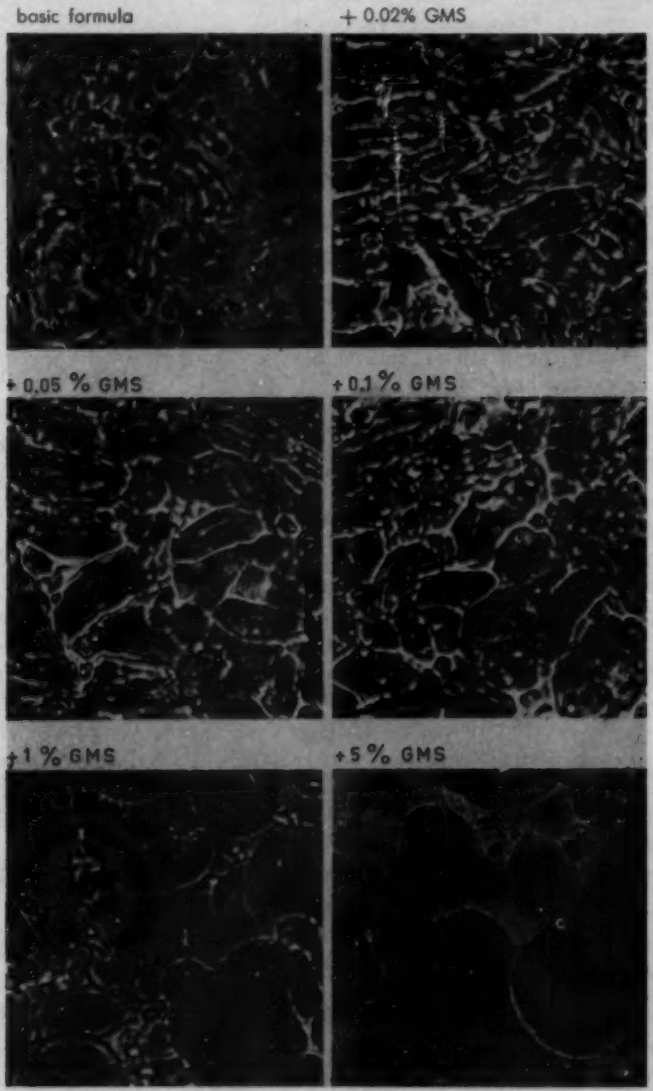


Fig. 3. Structure of cell walls in the crumb of starch bread. Magnification 500×.

was decreased so much that under the microscope the sections of bread crumb, embedded in glycerine, appeared to have fallen apart into many loose starch granules and only a few agglomerates.

Discussion

Terms with an accepted meaning in colloid chemistry, such as stability, flocculation, dilatancy, and plasticity, are used herein. For the definitions the reader is referred to Kruyt (5,6).

In a stable suspension, repulsive forces between the particles prevent their coagulation. A hydrophobic suspension such as that of starch usually acquires the required stability by virtue of an electric double layer around the particles. Dilute suspensions show sedimentation of the particles. In a stable suspension sedimentation is slow, and the particles arriving at the bottom of the vessel remain apart by the same forces that prevent their aggregation in the suspension. This entails the particles' rolling over each other until the closest packing is reached.

If by some cause the repulsive forces in a dilute stable suspension are replaced by attractive forces, aggregates are formed which sediment more rapidly, while the sediment is bulky because the particles preserve the haphazard positions in which they touched each other for the first time.

In dilute stable suspensions the viscosity as a rule is purely Newtonian; that is, the viscosity is independent of the rate of shear. In a concentrate stable suspension, however, the viscosity is Newtonian only for low rates of shear. At somewhat higher shearing stresses the viscosity increases as a consequence of the repulsion between the nearly densely packed particles. The deformation may lead to a continuous network of particles which have approached their neighbors so close that the repulsion between them is very high. This network greatly hampers further deformation, and as a consequence the suspension behaves almost as a solid. The phenomenon is called dilatancy. The approach of some particles to one another in the suspension with nearly dense packing must inevitably be accompanied by a separation of most particles. The volume occupied by the system and the water absorption, therefore, increase if dilatancy occurs. The repulsive forces between the particles are again able to disperse the locally formed clusters. Therefore these will disappear unless they are newly created at a sufficient rate. As a consequence, dilatancy will only show itself if the rate of deformation exceeds the reciprocal of the natural relaxation time of the clusters. It follows that dilatancy occurs only at relatively high stresses and also that it has

a temporary nature. The system immediately flows, when the stress which caused dilatancy ceases.

Flocculation can be promoted by the same causes in dilute and in concentrated suspensions. In a concentrated suspension the aggregation of particles which accompanies flocculation does not, however, result in phase separation as in a dilute suspension. The aggregates form a coherent network through the whole system, with junction points between the particles. As a consequence the volume and the water absorption of the system increase. In a flocculated suspension no dilatancy can occur, because it can never be as concentrated as a stable suspension. When a concentrated suspension passes from the stable into the flocculated state, it gains plasticity. Plasticity is characterized by the existence of a yield stress, that must be exceeded in order to cause any permanent or viscous deformation; the apparent viscosity decreases with increasing rate of deformation. Plastic systems are easily deformed and modeled, but retain their form when left to themselves.

Flocculation of Diluted and Concentrated Starch Suspensions by GMS. As has been mentioned, starch forms a stable suspension in water. In dilute suspensions this is shown by the relatively slow settling and the compact sediment. It has further been shown that in a 2% starch suspension, upon the addition of GMS, large aggregates are formed which give a voluminous sediment; in other words, the stable suspension is changed into a flocculated one. It can be assumed that the flocculation is caused by the adsorption of GMS molecules on the starch granules (possibly because of the strong attraction between the polar groups of the GMS molecules and the equally polar starch surface). Making use of the figures for the surface area of wheat starch given by Stamberg (11), it was calculated that if 0.1% GMS is uniformly spread over the starch surface, a layer of about 50A° is formed. As the length of a molecule of GMS is of about the same order (4), this points to monomolecular adsorption.

The concentrated starch suspension (starch dough) to which no GMS has been added shows its stable character by the occurrence of dilatancy at stirring. Also, the combination of the fluid character of the dough and the reasonable consistency measured by the farinograph can be considered as a consequence of stability. The deformation by mixing is apparently quick enough to cause the observed increase of viscosity.

The concentrated system is flocculated by the addition of GMS. In accordance with what has been said on the flocculation of concentrated

suspensions in general, this does not result in a phase separation as in the 2% starch suspension, but in the transformation of the stable concentrated suspension into a system with plastic properties. With small GMS additions the junction points formed between the granules will have a limited lifetime, and so the dough keeps a more or less fluid character. As has been mentioned, with increasing addition of GMS the dough acquires more and more the character of a solid paste, which points to the formation of a more permanent network structure.

In accordance with its transition from a stable into a plastic system, the starch dough, after the addition of GMS, shows a decreased mobility at small stresses (gravity) and at the same time an increased mobility at stresses which are high enough to raise the viscosity of the stable system. This last appears from the large drop in the consistency measured with the farinograph (Table II) and the smaller band width of the farinogram (Fig. 1) as a result of GMS addition.

Shape of the Farinograms. As Markley (7) has pointed out, the fact that after a rest period curves of the type of curve B in Fig. 1 are obtained, suggests that the system is thixotropic. However, a thixotropic system is a solid when it is in rest and gains mobility when it is stirred, whereas it was mentioned that in our starch-water mixtures without additions the reverse was true. These systems are not thixotropic, and they show dilatancy. In accordance with this, curve D was obtained at half speed after a rest period, which does not indicate thixotropy. With mixing at normal speed immediately after a period of slow mixing, curve F was obtained, which still more markedly has the type of shape shown by curve B. Moreover, the ratio between curves F and D (or E) was much smaller than 2, which together with the shapes of curve F and curve B indicates that at normal speed the system has a higher mobility than at half speed. The results seem to indicate that the diagram of mobility against mixing speed would show a minimum between half and normal mixing speed in the farinograph.

Perhaps the explanation is as follows. According to the accepted explanation for dilatancy, a network is built up from the starch granules under the influence of mixing, giving rise to a mechanical resistance. With increasing mixing speed the density of the structure decreases more and more, and it must be expected that if the density falls below a certain value, the system does no longer gain rigidity, but on the contrary becomes more vulnerable, resulting in an increase in mobility.

As the addition of GMS diminishes dilatancy, it will also diminish

the difference between the mobilities at half speed and at normal speed, which explains that the relative differences described for the first series of curves are shown in a much lesser degree by the second series in Fig. 1.

The Fermentation Process. During fermentation the starch dough begins to work under the influence of the tensions raised by the carbon dioxide evolved. The dense packing of the starch granules in the stable suspension gives the dough the ability to retain gas. Under the influence of the deformations caused by the growing gas bubbles, the occurrence of dilatancy, resulting in rigidity of the dough, may be expected. At the same time the formed clusters are easily dispersed as a consequence of the repelling forces, and, accompanied by a continuous formation and breakdown, the dough rises. It will, however, be clear that the texture of such a dough must be easily distorted and irregular. This also explains the coarse and irregular bread crumb which results after baking.

In the flocculated dough the coherence between the starch granules, together with the accompanying absence of dilatancy, enables the regular development, during fermentation, of a less vulnerable dough structure. Consequently the bread crumb also shows a fine and regular texture if GMS has been added to the dough.

Too large an adhesion between starch granules prevents their relative movements and, therefore, dough rise. Apparently this happens after the addition of 5% GMS, unless this addition is accompanied by a higher water content of the dough.

The Baking Process. During the baking process the solubility of the starch increases in the warmth of the oven. The starch granules absorb water and thereby lose their crystallinity. Gelatinization is limited by lack of water, and thus the starch granules acquire the properties of very concentrated amorphous gels. Then the granules are flexible and get an elongated shape as a result of the tensions present. The polarity of the starch surface will increase very much as a result of swelling. Hence, when cooling after baking, the decreasing solubility of the starch is accompanied not only by the formation of bonds between the molecules in the starch granules, but also by mutual binding of the starch granules. In the bread without GMS, the contact between the granules is very close and it is apparent from the microphotographs (Fig. 3) that they are mutually bound on all sides. The firm mutual binding results in extreme hardness of the crumb.

The addition of GMS proved to cause a loose bread crumb. Moisture determinations showed that the addition of GMS to the dough

did not cause a decrease, but even some increase of the moisture content of the bread crumb. So there is no evidence for a decreased gelatinization as a cause for the diminished coherence in the crumb. The probable explanation is, that the mutual binding of the starch granules in the crumb of starch bread is weakened greatly by orientated binding of GMS molecules. In accordance with this, the microphotographs of the bread crumb show a decreasing number of contact points between the starch granules with increasing additions of GMS. For the cell walls of bread with 0.05 and 0.1% GMS, the microscopic pictures suggest that the starch granules are linked together by three-dimensionally distributed junction points, resulting in a structure, less compact than in the absence of GMS, but still rigid enough to prevent the starch granules from getting the spherical form of the separate granules shown by the last photograph (5% GMS). With 1% GMS this rigidity apparently has decreased considerably.

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A FARINOGRAPH TECHNIQUE FOR MACARONI DOUGHS[1]

G. N. IRVINE, J. W. BRADLEY, AND G. C. MARTIN

ABSTRACT

A technique is described for studying the farinogram characteristics of macaroni doughs in the absorption range of 26.5 to 36.0%; this is the general range used in processing macaroni. With the 50-g. bowl attached to the farinograph, the balance linkage is set in the rear position (normal for 300-g. bowl), the instrument is zeroed, and the dampening is set to 0.8 seconds.

Macaroni doughs prepared from various types of wheat may be distinguished from one another by the farinograph. Variation of durum wheat grade has a distinct effect on farinograms. Farinogram characteristics of semolinas produced from durum wheats grown in different years are quite similar for a given variety, provided that protein content does not change significantly. Variation of protein content, temperature, absorption, or particle size produces a distinct change in farinogram characteristics.

Although the farinograph has been used extensively to determine characteristics of bread doughs at or near baking absorption (52 to 65%), and Brabender has studied semolina doughs at absorptions greater than 53%[2], no report on the use of the farinograph to determine macaroni dough characteristics, at normal absorptions of 26.5 to 36.0%, has come to the authors' attention. This paper describes adjustments to the farinograph required for study of macaroni doughs, and illustrates the effect of wheat type, variety, grade, protein content, absorption, temperature, and particle size on the farinogram characteristics. The results for each series are discussed in separate sections.

Materials, Equipment, and Method

The semolinas and farinas used in the study of wheat type, grade, and protein content were milled in the laboratory on an Allis-Chalmers Experimental Mill. The milling procedure has been described by Fisher and Meredith (3). This method produces a semolina of relatively uniform particle size. The semolina used in the study of absorption, temperature, and particle size was commercially milled to 55% extraction from a No. 2 Canada Western Amber Durum.

The 50-g. stainless-steel bowl was attached to the farinograph. The linkage was moved to the rear position (the 300-g. bowl setting) and the damper was screwed down to the bottom position. The farino-

[1]Manuscript received July 14, 1960. Paper No. 193 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg 2, Canada.

[2]Brabender, C. W. Application of rheological measuring techniques in the alimentary paste industry. C. W. Brabender Instruments, Inc., South Hackensack, N.J. Mimeo., pp. 1–4 (1955).

graph was then zeroed and the damper was set to give 0.8 seconds from 1,000 to 100 Brabender units. With these settings it is possible to obtain farinograph curves with semolina in the absorption range 26.5 to 36.0%. The farinograph used for this study has a speed of 59.3 r.p.m. and a side clearance of 0.030 in. between the blade edge and the bowl sides. A sample of 50.00 ± 0.01 g. (14.0% moisture basis) was placed in the bowl which was maintained at 30°C., except in the temperature study. The farinograph containing the sample was started and the desired amount of water, at the same temperature as the bowl, was introduced at the corner of the bowl. The farinograph was run 4 minutes after the peak had been reached.

Three terms are used throughout this paper to describe farinograms. Dough development time (DDT) is used as defined in *Cereal Laboratory Methods* (1). The term maximum consistency (MC) is used to designate the height in Brabender units of the center of the curve at the point of maximum consistency. Tolerance index (TI) is also used as defined in *Cereal Laboratory Methods* (1), except that the difference of curve height is measured 4 minutes rather than 5 minutes after maximum consistency.

For assessing gluten quality, glutens were washed out from semolina doughs using a Theby Gluten Washer. The washing solution is a sodium chloride-phosphate buffer of pH 6.5. Glutens are washed by machine for 12 minutes and finished off with 2 minutes' hand washing.

Results and Discussion

Effect of Grade. The various grades of Canada Western (CW) Amber Durum have different farinogram characteristics. Figure 1 shows the farinograms of four grades of Amber Durum wheat at their

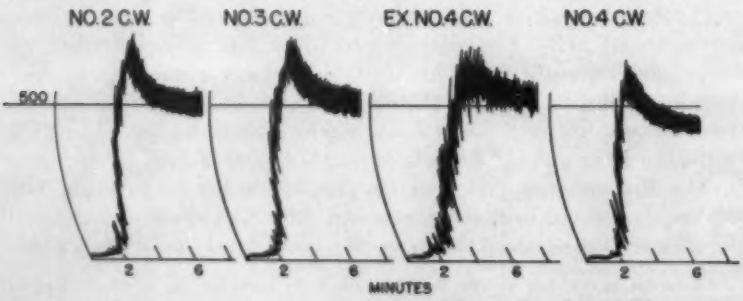


Fig. 1. Farinograms representing semolinas milled from various grades of Canada Western Amber Durum.

TABLE I

DATA FOR ABSORPTION AND DERIVED FARINOGRAM INDICES FOR SEMOLINAS MILLED FROM FOUR GRADES OF CANADA WESTERN AMBER DURUM

GRADE	ABSORPTION	DOUGH DEVELOPMENT TIME	MAXIMUM CONSISTENCY	TOLERANCE INDEX
	%	*minutes*	*B.u.*	*B.u.*
No. 2 CW	31.5	4.00	665	165
No. 3 CW	31.5	4.25	650	140
No. 4 CW	33.5	4.25	570	130
Extra No. 4 CW	32.5	5.25	580	55

processing absorption as determined by the micro procedure for macaroni processing (4). As grade decreases (Table I), dough development time increases and tolerance index decreases slightly. Maximum consistency also appears to decrease but this is largely due to increased absorption. The small tolerance index for Extra No. 4 CW will later be shown to be a varietal characteristic of Pelissier. This variety is excluded from the top grades of Amber Durum wheat (Nos. 1, 2 and 3 CW) and thus enters the next grade, Extra No. 4 CW.

Effect of Variety. Semolinas milled from the standard varieties Mindum, Stewart, Carleton, Golden Ball, and Ramsey produce similar farinograms. Pelissier is the only licensed durum variety grown in Canada that produces a different farinogram. Figure 2 shows the typical curve of this variety compared with Mindum at the same absorption of 33.0%. The dough development time for Mindum was 3.25 minutes as compared with 10.0 minutes for Pelissier. The maximum

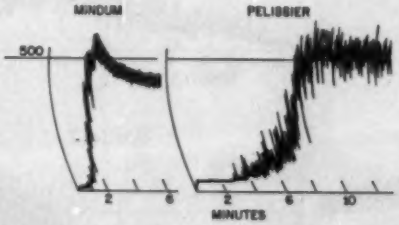


Fig. 2. Farinograms representing Mindum and Pelissier at 33.0% absorption.

consistency and tolerance index for Mindum were 545 B.u. and 125 B.u.; for Pelissier 530 B.u. and 20 B.u. The low tolerance index of Pelissier changes very slightly with varying absorption. This characteristic is related to gluten properties, not grade or absorption.

A low tolerance index, of 75 B.u. or less, is shown by certain durum varieties and other wheat types which have the following semolina

gluten properties as determined from the washed-out gluten. The gluten is elastic to very elastic, extensible, and forms a strong, fairly thin membrane on sheeting. These glutens would be classed as bread wheat types. Durum glutens from the standard Canadian varieties are less elastic, more extensible, and form a fairly weak, very thin membrane.

Figure 3 shows the farinograms of macaroni doughs of four foreign durums, made at processing absorption. These durums were exported

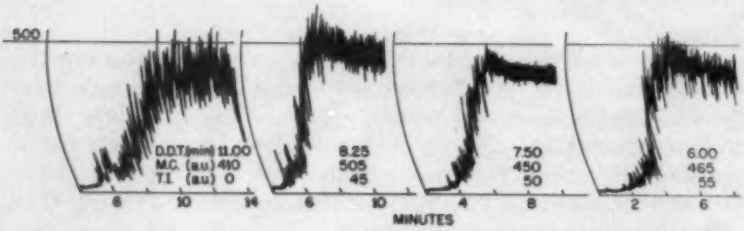


Fig. 3. Farinograms representing foreign durum macaroni doughs at 35.0% absorption.

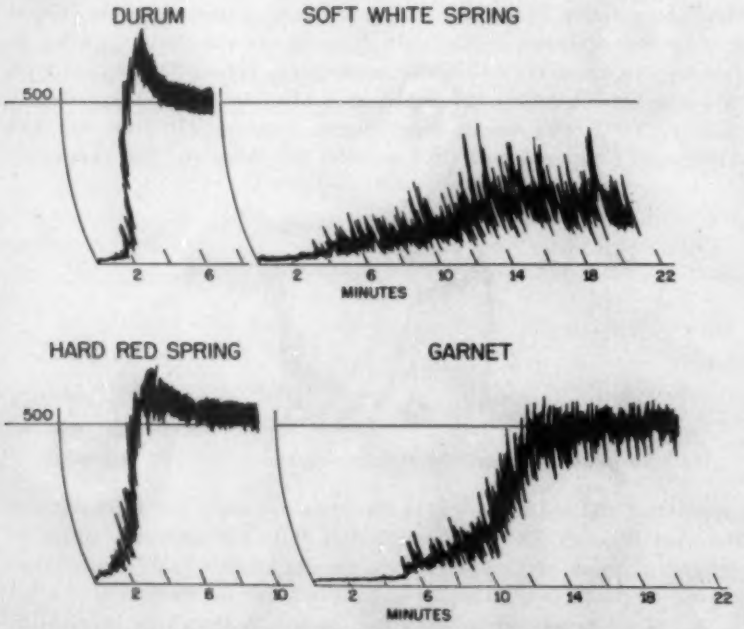


Fig. 4. Farinograms representing various types of wheat.

from four countries and are representative only of individual cargoes. The first and last farinograms represent the same variety grown in two different countries. The varieties of the other two samples are unknown. The grade of these wheats as determined by the Board of Grain Commissioners Inspection Branch ranged from No. 2 to No. 3. This series illustrates further the wide variation in farinogram characteristics which may occur with durums grown in widely differing environments.

Effect of Wheat Type. Farinas, milled from various types of wheat, produce farinograms which are considerably different from farinograms of durum semolinas. Figure 4 shows the farinograms for Soft White Spring, Garnet, and Hard Red Spring compared with a No. 2 CW Amber Durum curve at constant absorption of 31.5%. Samples which graded either No. 2 or No. 3 were used to reduce possible effects of differences in grade. The absorption used is not a macaroni-processing absorption, but was selected to show differences that exist at constant absorption.

Effect of Protein. The farinograms shown previously represent samples which vary in protein content. To study the effect of protein

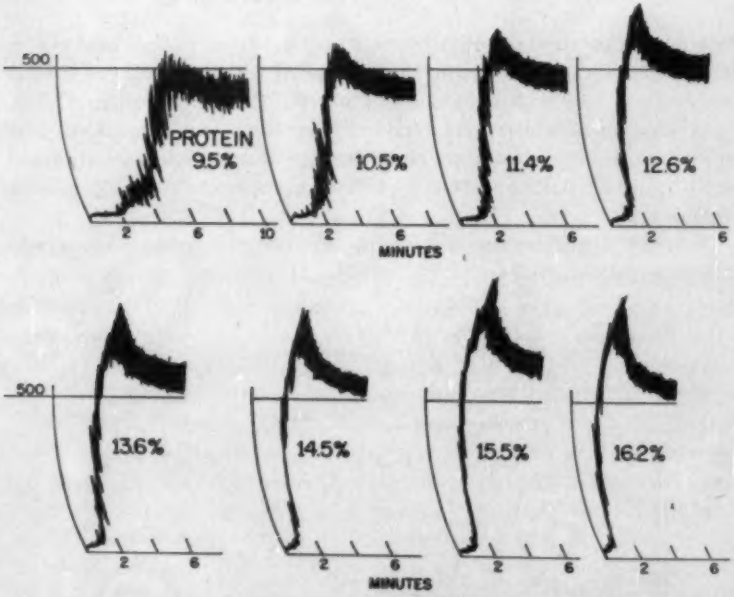


Fig. 5. Effect of protein content on farinogram characteristics.

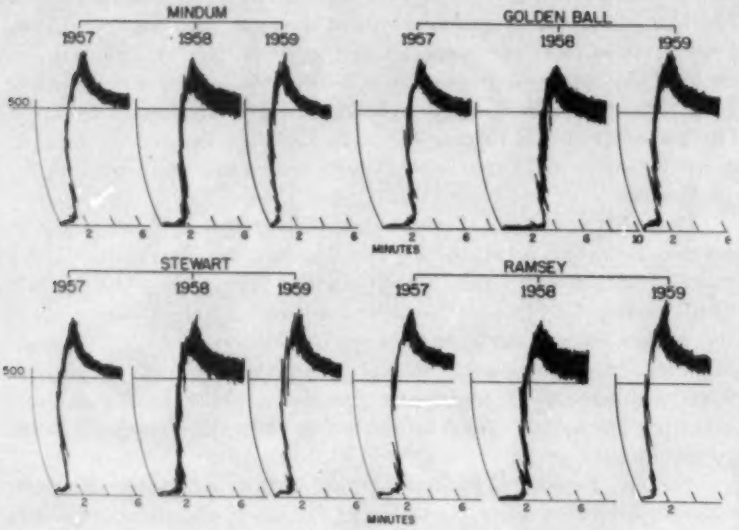


Fig. 6. Farinograms of four varieties grown in three different years.

content independent of the influence of wheat type, variety, and grade, two samples of Stewart durum were obtained which graded No. 2 CW. One sample had a protein content of 10.5% and the other 17.5%. Eight samples of wheat were blended from these two samples, so that on milling to 50% extraction, eight samples of semolina were obtained which ranged from 9.5 to 16.2% protein in approximately 1% protein increments.

Figure 5 illustrates the effect of protein content on the farinograms of macaroni doughs at 31.5%. Table II contains the farinogram

TABLE II

EFFECT OF PROTEIN CONTENT ON FARINOGRAM CHARACTERISTICS OF MACARONI DOUGHS

Protein Content	Dough Development Time	Maximum Consistency	Tolerance Index
%	*minutes*	*B.U.*	*B.U.*
9.5	6.25	460	30
10.5	4.50	520	80
11.4	4.00	570	100
12.6	3.25	645	135
13.6	3.00	710	140
14.5	2.25	710	155
15.5	2.50	800	185
16.2	2.25	760	220

measurements for this series. As protein content increases, dough development time decreases, maximum consistency increases, and tolerance index increases. The farinogram of a very low protein sample has the same characteristics as the farinogram of a poor variety or a nondurum wheat.

Effect of Environment. Macaroni doughs of four standard varieties (Mindum, Golden Ball, Stewart, and Ramsey) have been studied in the farinograph for the years 1957, 1958, and 1959. Each variety sample represented a composite from a number of experimental stations in Western Canada. Figure 6 shows the farinograms of these varieties at 31.5% absorption. Table III contains the farinogram data, and shows the protein and wet gluten contents of the samples.

TABLE III

THE PROTEIN CONTENT, WET GLUTEN CONTENT, AND FARINOGRAM CHARACTERISTICS OF SEMOLINAS FROM FOUR VARIETIES OF CANADIAN AMBER DURUM GROWN IN DIFFERENT YEARS

VARIETY	YEAR GROWN	PROTEIN CONTENT	WET GLUTEN CONTENT	DOUGH DEVELOPMENT TIME	MAXIMUM CONSISTENCY	TOLERANCE INDEX
		%	%	*minutes*	*B.U.*	*B.U.*
Mindum	1957	13.8	40.4	3.00	685	160
	1958	12.1	35.6	4.00	640	125
	1959	13.7	41.6	2.50	670	170
Golden Ball	1957	12.6	36.1	4.25	660	140
	1958	11.6	33.9	5.50	630	130
	1959	12.7	37.7	3.50	675	145
Stewart	1957	13.7	39.7	2.75	680	170
	1958	12.1	36.2	4.25	670	130
	1959	13.8	42.6	3.00	710	170
Ramsey	1957	14.0	40.8	3.50	730	150
	1958	12.3	37.7	4.50	670	115
	1959	13.8	42.7	3.00	740	160

The farinogram characteristics of each variety are quite similar for 1957 and 1959, but are slightly different for 1958. In 1958 dough development time increased, maximum consistency decreased, and tolerance index decreased slightly. In the 1958 samples, protein content was lower than in the samples grown in 1957 and 1959. Because of the effect of protein content discussed previously, it was concluded that the variation in farinogram characteristics from year to year was due largely to a variation in protein content. Minor differences between farinograms for years of equal protein (1957 and 1959) are probably due to differences in gluten quality as shown by a change in wet gluten content. Year-to-year variations in farinogram characteristics for a given variety are small compared to variations due to wheat type, variety, and grade.

Effect of Absorption. Farinograms in Fig 7 show the effect of varying absorption on a top-grade commercial semolina. As absorption increases, dough development time decreases, maximum consistency decreases, and tolerance index does not change significantly.

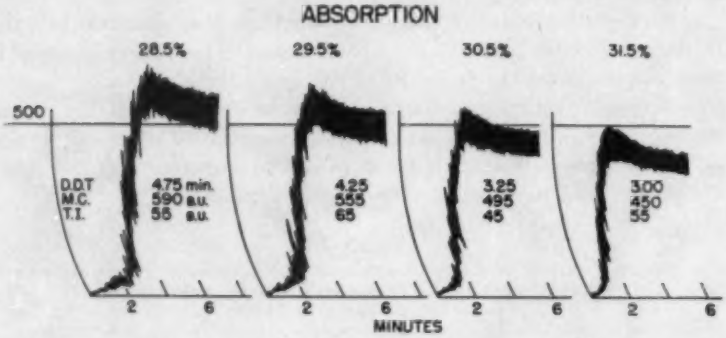


Fig. 7. Effect of varying absorption on farinograms of commercial semolina at 30°C.

The low tolerance index is not due to variety, type, protein, or grade, as the semolina used was milled from a No. 2 Canada Western Amber Durum. It will be shown later that the low tolerance index is caused by the presence of large particles in the semolina.

Effect of Temperature. Farinograms of commercial semolina were obtained using a constant absorption of 28.5% while controlling the mixing bowl temperature at 30°, 35°, and 40°C. The farinograms are shown in Fig. 8. As temperature increases, dough development time decreases, maximum consistency decreases, and tolerance index increases. It is therefore necessary to control the temperature of the bowl to evaluate farinograms.

Effect of Particle Size. The farinogram (extreme left, Fig. 9) of the

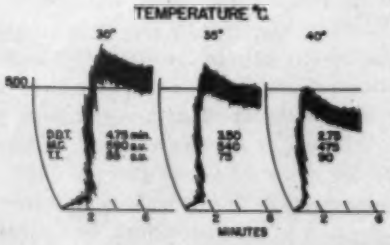


Fig. 8. Effect of varying temperature on farinograms of commercial semolina at 28.5% absorption.

commercial semolina milled from a No. 2 CW Amber Durum does not look like the farinogram of a macaroni dough produced from semolina milled in the laboratory. The dough development time of 3 minutes is normal, but the low maximum consistency of 450 B.u. and the low tolerance index of 55 B.u. are not consistent with the characteristics of a No. 2 CW Amber Durum semolina.

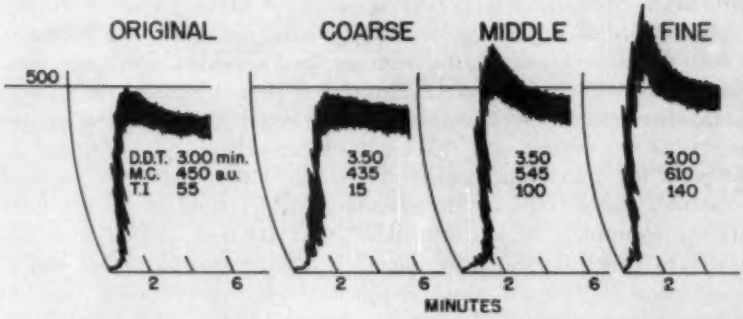


Fig. 9. Effect of varying particle size on farinograms of commercial semolina at 31.5% absorption.

Table IV shows the relative proportions of various-sized particles in a commercial semolina as compared to the laboratory-milled semolina. A sample of this commercial semolina was separated into three fractions by sieving. The fraction retained by a 40-mesh†[3] was designated as the coarse fraction; the fraction which passed through the 40-

TABLE IV
RANGE OF PARTICLE SIZE IN SEMOLINAS USED

SAMPLE	HELD ON 40-MESH [a]	HELD ON 80-MESH [a]	PASSED THROUGH 80-MESH [a]
	%	%	%
Laboratory milled	0	92	8
Commercial	28	66	6

[a] U.S. Standard Sieve series.

mesh† and was retained by the 80-mesh† was designated as the middle fraction; and the fraction which passed through an 80-mesh† was designated as the fine fraction. Figure 9 shows the farinograms of the original semolina and the three fractions, all run at a constant absorption of 31.5%.

As particle size decreases, maximum consistency increases and

[3] † Designates U.S. Standard Sieve series.

tolerance index increases. Dough development time does not change significantly. These results seem to show that the low maximum consistency and low tolerance index of the original sample are due to the presence of large particles in the semolina.

The interrupted farinograph technique for bread doughs (2) has been described by Brabender. This technique has been used in this laboratory to study semolinas at lower absorptions. The curve produced after the rest period is identical to the original curve, independent of variety, grade, and protein content for a homogeneous semolina. However, the heterogeneous semolina which contains large particles will, with a 15-minute rest period, change its farinogram characteristics to resemble a homogeneous semolina. The major effects of rest period (Fig. 10) on the heterogeneous semolina are to increase the maximum consistency (from 470 to 545 B.u. in the example) and to increase the tolerance index (from 30 to 120 B.u. in the example). It was concluded that the rest period enabled water to penetrate the large particles. This redistribution of water produces a farinogram which is similar to the curve shown for the homogeneous semolina. It may be that this interrupted technique, despite the greater time required, will prove a useful modification for general studies of commercial semolinas.

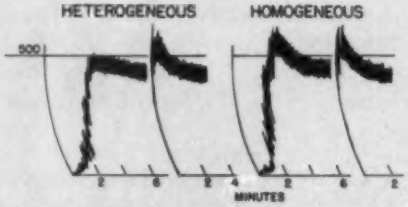


Fig. 10. Interrupted farinograms on commercial semolina.

The Combined Effect of Absorption, Temperature, and Particle Size. It has been shown that increasing absorption and increasing temperature have the same effect on dough development time and maximum consistency. Figure 11 shows that farinograms can be reproduced at various absorptions by adjusting the temperature to give the same dough development time of 4.75 minutes and maximum consistency of 590 B.u. However, the tolerance index shows the same increase from 55 to 90 B.u. as that obtained at a constant absorption with the same temperature changes (Fig. 8). It therefore appears that tolerance index is independent of absorption. This finding agrees closely with the results of the absorption study (Fig. 7).

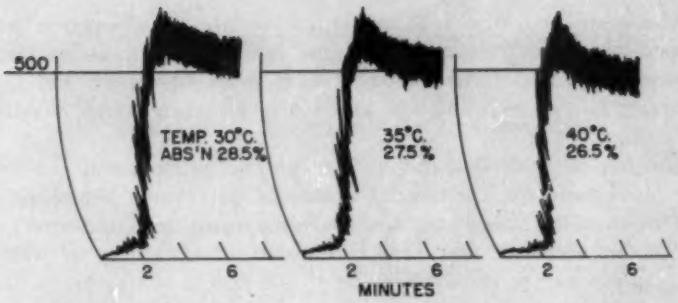


Fig. 11. Combined effect of temperature and absorption on farinograms.

A farinogram that resembles the curve of a homogeneous semolina dough can be produced from a heterogeneous semolina by varying absorption and temperature. Figure 12 shows a No. 2 commercial semolina curve produced at normal temperature (30°C.) and absorption (31.5%) compared to a curve produced at 40°C. and 26.5% absorption.

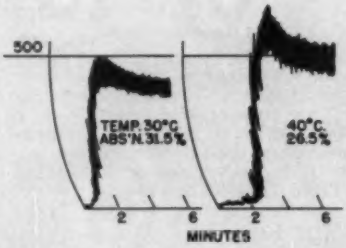


Fig. 12. Farinograms showing how curve characteristics may be controlled by absorption and temperature.

General Discussion

Farinograms of macaroni doughs were obtained using macaroni processing absorptions and appropriate settings for the farinograph. The curve characteristics were influenced to a greater extent by wheat type and durum variety than by grade and protein content.

The two principal types of farinograms were those with a high tolerance index and those with a low tolerance index. High tolerance index values are associated with good durum varieties, reasonably high protein content, and homogeneous particle size; low values are obtained from wheats other than durums, from low-protein samples, from poor durum varieties, and from heterogeneous semolina.

Maximum consistency is influenced mainly by absorption and temperature. Dough development time is influenced most markedly by wheat type and variety but also varies with absorption and temperature; low protein and low grade may lengthen dough development time.

It seems probable that the farinograph may prove useful as a control instrument for commercial macaroni processing. Semolina of uniform quality has always been advantageous, and uniformity is even more necessary now that continuous processing is so widely used. In essence the problem relates to the degree of correlation that exists between the processing properties of semolina and the characteristics of the farinograms. This correlation is not readily examined in a laboratory equipped only for processing semolina on a microscale. Nevertheless, the results presented in this paper do suggest that changes in various semolina qualities are reflected by typical changes in farinograms and suggest that this technique could be developed to provide a means of controlling paste properties in continuous macaroni processing plants. Further work along these lines is being done in this laboratory.

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SOME VOLATILE CARBONYL COMPOUNDS ARISING DURING PANARY FERMENTATION[1]

F. E. KOHN[2], L. WISEBLATT, AND L. S. FOSDICK[3]

ABSTRACT

Volatile carbonyl compounds produced during dough fermentation were isolated and identified. Straight doughs from normal ingredients and from ingredients specially treated to attain minimal bacterial populations were compared as to the types and amounts of carbonyl compounds recoverable. The compounds identified in both normal and low-bacteria doughs are acetone, acetaldehyde, pyruvaldehyde, n-hexanal, n-butyraldehyde, isovaleraldehyde, and 2-hexanone. Benzaldehyde, which was identified in normal doughs, was not detected in low-bacteria doughs. Quantitative estimates of these substances showed no notable order-of-magnitude differences between the doughs. These compounds are not detectable in the ingredients, and are clearly fermentation by-products.

The importance of panary fermentation in determining the flavor and aroma of white pan bread has been well established by the investigations of Baker and associates (1,2). These workers have shown that unfermented doughs are incapable of producing products having a flavor resembling bread derived from fermented doughs.

Baker has attempted to account for this in terms of yeast action upon amino acids and sugars, resulting in the formation of fusel oils and "browning" precursors respectively. Other investigators (5) have implicated volatile products formed by the action of the bacterial flora known to be present in compressed yeast, flour, and other ingredients of doughs.

In the light of present knowledge of the volatile carbonyl constituents recoverable from fresh bread (6) it was considered of potential value to determine whether any of these compounds actually originated during fermentation. If possible, any compounds identified would also be related either to the action of yeast or normal bacterial flora, or to both.

Materials and Methods

Dough Formulation. The following formula was used in the preparation of all doughs.

[1] Manuscript received May 5, 1960. Presented at the 45th annual meeting, Chicago, Illinois, May 1960. Contribution from the American Institute of Baking, Chicago, Illinois. This research was supported by a grant from the Max C. Fleischmann Foundation.

[2] Present address: Industrial Bio-Test Laboratories, Northbrook, Illinois. This paper is taken from a portion of a thesis submitted by F. E. Kohn to the Graduate School of Northwestern University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Professor of Chemistry, Northwestern University School of Dentistry.

	g
Flour (nonbromated)	1,000
Water	700
Yeast, compressed	30
Salt	20
Glucose	60
Nonfat dry milk	40
Lard	30

Fermentation Conditions. Doughs were no-punch straight doughs, fermented for 4 hours at a temperature of 24°C. and relative humidity of 85%.

Pure Yeast. The pure yeast culture had a total bacteria count of 20 per g.

Sterile Ingredients. Nonfat dry milk and flour were sterilized with ethylene oxide. Lard was autoclaved 15 minutes at 121°C. Sugar and salt solutions and water were subjected to Seitz filtration. After these treatments the ingredients were found to be sterile.

Recovery of Volatiles from Doughs. During fermentation, all evolved gases were aspirated under mild vacuum through a train of three gas scrubbing columns, each containing 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid, to trap any volatile carbonyl compounds which might otherwise be lost. By allowing fresh air to pass across the entire dough surface on its way to the gas scrubbers, such carbonyls were trapped very effectively. The recovered hydrazones were combined with those obtained from the vacuum strippings.

At the completion of the desired fermentation period, 2,800 ml. of carbonyl-free isopropanol were added to the dough (final alcohol concentration approximately 80% by volume) to precipitate the proteins. This resulted in a granular precipitate which was easily separated from the medium by centrifugation and subsequent decantation of this supernatant liquid. The solids were then washed with three 50-ml. portions of carbonyl-free isopropanol and finally with two 50-ml. portions of distilled water. All washings were combined with the original supernatant liquid.

The system for recovering the volatiles consisted of a rotary flash evaporator connected to a vacuum pump through a series of four cold traps. Three of these traps were chilled by dry ice and alcohol mixtures; the final trap was chilled with liquid nitrogen. The pump was fitted with a needle valve which acted as a bleed mechanism to allow precise pressure control during the distillation. The pressure was maintained in the region of 50 to 200 mm. mercury throughout most of the distillation. After complete removal of the aqueous alcohol phase, the operating pressure was reduced to 50 μ mercury and maintained at this level for 30 minutes. After this treatment

the residue was devoid of any discernible odor.

Derivatization and Separation of Carbonyl Compounds. The condensate collected in the cold traps was treated with an excess of 2,4-dinitrophenylhydrazine in hydrochloric acid sufficient to bring the final acid concentration to approximately 2*N*. The dinitrophenylhydrazones (DNPH) were allowed to form during 24 hours at room temperature.

The isopropyl alcohol was then removed by flash evaporation at 40°C. and the acid was neutralized with sodium bicarbonate. Water was next removed by flash evaporation and the DNPH's were completely extracted with warm benzene. This extract was concentrated, then treated with four volumes of petroleum ether to precipitate the dicarbonyl compounds and excess reagent, then filtered. The filtrate was separated into its components by a combination of chromatography on a silicic acid column and paper chromatography, using decalin and dimethylformamide. This procedure has been described in detail (6).

Identification of Carbonyl Compounds. The DNPH's were next recrystallized from aqueous ethanol to yield products suitable for melting-point determinations.

Quantitative Determinations. For quantitative determinations, known aliquots of the components separated by column chromatography were stripped of solvent, dissolved in benzene, and purified further, if necessary, by paper chromatography. The resolved fractions were eluted quantitatively from the paper with warm benzene, cooled, and diluted to volume. Quantitative determinations were made by ultraviolet spectrophotometry at 360–370 mμ, using the findings of Jones, Holmes, and Seligman (3) that all saturated carbonyl hydrazones have essentially the same molar absorbancy and that the absorption of hydrazone solutions follows the Lambert-Beer law.

Testing of Ingredients. Samples of the ingredients were examined for the presence of volatile carbonyl compounds by distilling alcoholic extracts and testing the distillates with 2,4-dinitrophenylhydrazine solution followed by alcoholic alkali, according to Lappin and Clark (4). In all cases, the results were essentially negative, so that those carbonyl compounds reported here can be considered as fermentation by-products.

Results and Discussion

The volatile compounds isolated from the dough made with commercial yeast and nonsterile ingredients, together with their melting

points and melting points of mixtures with equal quantities of pure reference derivatives, are listed in Table I. The amount of pyruvaldehyde was very small. In addition, a small amount of a component was found whose DNPH melted at 120°–122°C. This did not correspond to any available reference DNPH and the compound could not be identified.

TABLE I

VOLATILE CARBONYL COMPOUNDS FORMED IN THE FERMENTATION OF DOUGH

DESCRIPTION	M.P.	CORRESPONDING REFERENCE HYDRAZONE	M.P.	MIXED M.P.
	°C		°C	°C
Yellow-orange rods	106–107	2-hexanone	107	106–107
Yellow needles	102–103	n-hexanal	107	103–105
Orange rods	115–116	isovaleraldehyde	120	117–118
Orange-yellow plates	115	n-butyraldehyde	122	116–117
Yellow needles	125	acetone	127	126
Deep orange needles	238	benzaldehyde	243	241
Yellow needles	155–157	acetaldehyde	163	157–159
Deep orange needles	292	pyruvaldehyde	308	300–306

Acetaldehyde, acetone, 2-hexanone, and pyruvaldehyde have also been reported in bread (6). The remaining compounds in Table I may have reacted during baking, or volatilized in the oven, to be absent in bread.

The effects of the bacteria normally present in the yeast and other ingredients on the formation of carbonyl compounds during fermentation were studied both qualitatively and quantitatively. For this purpose the ingredients used in the dough formula, except yeast, were sterilized. Yeast with a very low bacteria count was used. The bacteria count in the yeasts and doughs is given below.

	Bacteria per g. Control dough	*Low-bacteria dough*
Yeast	1.3×10^8	20
Dough after fermentation	52×10^8	1,200

To ensure that doughs made with sterile ingredients and yeast of low bacteria count were not abnormal, they were baked into bread. The bread was comparable in quality characteristics with that made from the control dough.

The same volatile compounds were obtained from the fermentation of the low-bacteria dough as were obtained from the control dough (Table I). Pyruvaldehyde and benzaldehyde were not determined quantitatively because the amounts present were too small for accurate analysis. Because the DNPH's of 2-hexanone and n-hexanal

could not be separated quantitatively in pure form they were determined together. Quantitative determinations were made on duplicate doughs and the results shown in Table II are the averages.

TABLE II

AMOUNTS OF CARBONYL COMPOUNDS FORMED IN FERMENTATION OF NORMAL DOUGH AND ONE OF LOW BACTERIAL CONTENT

COMPOUND	NORMAL DOUGH	LOW-BACTERIA DOUGH
	ppm	*ppm*
2-Hexanone } n-Hexanal }	246	263
Isovaleraldehyde	24	22
n-Butyraldehyde	22	25
Acetone	70	49
Acetaldehyde	33	15

There was a considerable difference in results between duplicates, so that the data of Table II should be interpreted as giving only the order of magnitude. Since Table II shows no differences in the orders of magnitudes of the individual compounds, the normal bacterial flora of the fermenting dough apparently have little effect on the relative amounts of the carbonyl compounds formed. These compounds appear to be essentially the results of yeast fermentation.

Acknowledgments

Grateful acknowledgment is made to the following: Red Star Yeast and Products Co. for supplying the low-bacteria yeast; The Griffith Laboratories, Inc., for sterilizing ingredients with ethylene oxide; and Karl J. Zobel, A.I.B. research bacteriologist, for determining the bacterial content of ingredients and doughs.

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THE CONTROL OF FUNGI DURING THE MALTING OF WHEAT[1]

JAMES R. FLEMING, JOHN A. JOHNSON, AND BYRON S. MILLER[2]

ABSTRACT

Low levels of formaldehyde or 8-hydroxyquinoline sulfate completely inhibited growth of fungi on wheat during malting. Mercuric chloride, methylmercuricyandiamide, and *o*-phenylphenate (Dowicide) also were effective for control of fungi. Tetrachloro-*p*-benzoquinone, n-propyl vanillate, 2-thiobarbituric acid, thiourea, n-trichloromethylthiotetrahydrophthalimide, and several other substances were more effective than potassium permanganate and the hypochlorites. It is likely that many of these chemicals cannot be used in malting because of their toxicity to animals and humans.

Formaldehyde used in 0.05% concentration in the steep liquor during the last 1 to 6 hours of the steeping period completely controlled growth of fungi during germination. The amount of formaldehyde retained in the finished malt was as little as 0.003 p.p.m. This amount of formaldehyde did not inhibit yeast fermentation and would probably be insignificant so far as human consumption of bread is concerned.

The role of microorganisms during malting is unknown, but their development is generally not desired. They have been shown to be present both externally and internally (6). Reduction of germination (14,15), development of unusual flavors and of materials which inhibit yeast fermentation (14), water sensitivity of some barleys (2), and production of "gushing" beer (4) are among the undesirable conditions attributed to microorganisms which grow during malting of barley.

Comparatively little information concerning control of fungi during malting is available. Calcium and sodium hypochlorites, sodium hyposulfite, hydrogen peroxide, and potassium permanganate have been used commercially (13) to control fungi. Formaldehyde was one of the first chemicals used as a seed protectant. This treatment normally reduced seed germination, but Braun (3) found that presoaking of grains prior to formaldehyde treatment materially reduced the inhibitory effect on germination. Hurd (12) has shown that formaldehyde was absorbed very slowly by wheat and that it was dissipated gradually when wheat was stored moist. Recently

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[2] Instructor and Professor, respectively, Department of Flour and Feed Milling Industries, Kansas State University; and Chemist, Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture.

Piratzky (18) and Grimm and Baker (11) have advocated the use of formaldehyde to treat barley prior to malting.

The mold problem during the malting of wheat is thought to be greater than that with barley which may contain certain growth inhibitors in its husks (13). Difficulty in germinating some wheats for more than 6 days without excessive mold developing has been experienced by the writers. The object of this study was to investigate chemical means to control fungi during malting of wheat.

Materials and Methods

The malted wheats used were produced by using laboratory-scale malting equipment assembled for an earlier investigation (9). It consisted of a refrigerated steep tank, a germination chamber, and a forced-draft convection drying oven. All units were equipped with controls for close regulation of humidity and/or temperature. The malts were prepared by steeping wheats to 42% moisture, germinating at 62°F., and drying at 104°F. for 24 hours. Fifty-gram portions of wheat were used.

The seed treatments were of two types. The first consisted of steeping grain for various times in solutions of chemicals being examined. The second consisted of dusting the grain, after steeping, with powders in which wheat starch was used as the carrier for the chemicals to be applied. The powders were prepared by drying (in a rotary evaporator) a suspension of starch in an alcoholic solution of the chemicals. A 2.5-g. portion of powder was applied to each 50 g. of wheat. The substances and the concentrations employed in both techniques are given in Tables I and II.

Effectiveness of the chemical treatments was evaluated by an agar plate technique (6) to demonstrate the existence of molds in cereal grains. Incubation time was 3 days at 30°C.

Alpha-amylase activity was determined by the method of Sandstedt *et al.* (21) as modified by Redfern (19). Protease activity was measured by the method of Miller (16).

Residual formaldehyde in the malted wheats was determined by a colorimetric technique employing chromotropic acid (5). The effect of small amounts of formaldehyde added to flour on the rate of yeast fermentation was studied by measuring gas production (1).

Results and Discussion

Chemicals Ineffective or Only Partially Effective in Controlling Fungi. The following chemicals were investigated and found to be ineffective in controlling molds and, in addition, many reduced seed

TABLE I

EFFECT OF CERTAIN CHEMICALS ON THE DEVELOPMENT OF FUNGI AND GROWTH OF ACROSPIRES OF TRIUMPH WHEAT DURING MALTING

CHEMICAL[a]	CONCENTRATION	FUNGUS DEVELOPMENT[b]	ACROSPIRE DEVELOPMENT[c]
	%		
Control		+++++	+++++
1-acetyl-2 thiohydantoin	0.10	+++	+++++
	0.20	+++	+++++
Ethyl vanillate	0.20	++++	+++++
Calcium hypochlorite	0.10	++++	+++++
	0.20	++++	+++++
2-hydroxy-3-hexenoic acid delta-lactone, sodium salt (Dehydrochloroacetic acid, sodium salt)	0.05	++++	+++++
	0.10	+++	+++
Isobutyl vanillate	0.10	++++	+++++
	0.20	+++	+++++
2-mercaptobenzothiazole	0.10	++++	++++++
	0.20	+++	+++++
Paraformaldehyde	0.05	++++	+++
	0.10	+++	+++
n-Phenyl-2,4-thiazolidinedione	0.10	+++	+++++
	0.15	+++	+++
1-Phenyl-3,5-dimethyl-4-nitrosopyrazole	0.05	++++	+++++
	0.10	+++	+++
	0.15	++	+++
Potassium permanganate	0.01	++++	+++++
	0.03	++++	+++++
	0.05	+++	+++++
	0.10	+++	+++
Potassium sorbate	0.20	++++	+++
n-Propyl vanillate	0.10	+++	+++++
	0.20	++	+++
Sodium bisulfite	0.20	++++	+++
Sodium hypochlorite	0.10	++++	+++
	0.20	++++	+++++
Sorbic acid	0.07	++++	++
	0.10	++++	++
Tetrachloro-*p*-benzoquinone (Chloranil)	0.10	+++	+++++
	0.20	++	++++++
2-Thiobarbituric acid	0.05	++++	+++++
	0.10	+++	+++++
Thiourea	0.10	++++	+++++
	0.20	+++	++++++
n-Trichloromethylthio-tetrahydrophthalimide (Captan)	0.05	++++	+++++
	0.10	+++	+++++
Zinc trichlorophenate	0.025	++	+++++
	0.05	+	+++
Zinc ethylenebisthiocarbamate	0.025	+++	+++++
	0.05	++	+++++

[a] Applied as powder except for 0.10% potassium permanganate and 0.10% sodium hypochlorite which were added to the steepwater 6 hours prior to the end of the steeping period. The application of some of these chemicals in powder form was necessary because of their insolubility. Application in the steepwater is to be preferred because of its convenience and uniformity.

[b] Five plus signs indicate mold growth equivalent to that of the control. Fewer plus signs indicate progressively less fungal growth.

[c] Five plus signs indicate acrospire development equivalent to that of the control. Fewer plus signs indicate progressively less and more plus signs indicate progressively greater acrospire growth.

germination: acetaldehyde, acetic acid, acrolein, *p*-aminobenzoic acid, benzoxazolinone, caffeic acid, sodium *p*-toluene sulfone chloramide (chloramine T), crotonic acid, 3-chloropropionic acid, copper sulfate, 2-ethoxyethyl acetate, formic acid, furfural, diacetyl, lactic acid, phenothiazine, phenylacetic acid, potassium sorbate, propionic acid, propionaldehyde, propylene glycol, propylene glycol dipropionate, and Sucrodet D-608 (a sucrose dipalmitate).

Treatments partially effective in controlling fungi are given in Table I. The agar plate method (6) of evaluating these treatments is more sensitive than observations on the appearance, smell, or taste of the malt. With the agar plate technique, mold growth could be readily observed after 3 days of incubation, whereas in wheat malting, mold proliferation could not be discerned readily until after 4 to 5 days of germination.

The hypochlorites, sodium bisulfite, and potassium permanganate treatments were found to be among the least effective of the substances studied (Table I). They tended, however, to stimulate acrospire growth as indicated by Hopkins and Krause (13).

Ethyl vanillate, tetrachloro-*p*-benzoquinone, 2-mercaptobenzothiazole, and thiourea, when applied in powder form following the steep period, reduced mold growth slightly. The last two appeared to stimulate acrospire growth slightly, but both contributed unusual odors to the malt.

Paraformaldehyde reduced both mold and acrospire growth significantly when applied in dry form at 0.10% concentration. Crocker and Barton (7) have reported that seed treatment with formaldehyde may be injurious because of the formation of the paraformaldehyde by polymerization of formaldehyde.

n-Trichloromethylthio-tetrahydrophthalimide, sodium dehydroacetate, 2-thiobarbituric acid, n-phenyl-2,4-thiazolidinedione, tetrachloro-*p*-benzoquinone and 1-phenyl-3,5-dimethyl-4-nitrosopyrazole all significantly reduced mold growth without markedly reducing acrospire development. These substances, however (with the possible exception of sodium dehydroacetate), are of little practical value because of their toxicity. The esters of vanillic acid were more effective in controlling fungi than were the hypochlorites and have been found to be relatively innocuous (8). However, they possess strong aromas which may preclude their use. Sorbic acid and potassium sorbate, now being used as food preservatives, were found to be of no value in malting for they reduce the rate of germination markedly when used at concentrations high enough to affect the growth of fungi.

Chemicals Effective in Controlling Molds. Data concerning the

TABLE II

EFFECT OF CERTAIN CHEMICALS ON THE DEVELOPMENT OF FUNGI, GROWTH OF ACROSPIRES, AND ENZYME ACTIVITIES OF TRIUMPH WHEAT, DURING MALTING

CHEMICAL	TIME PRESENT IN STEEP-WATER	CONCENTRATION	FUNGUS DEVELOPMENT[a]	ACROSPIRE DEVELOPMENT[b]	ALPHA-AMYLASE ACTIVITY[c]	PROTEASE ACTIVITY[d]
	hours	%			*SKB units/g*	*HU/g*
Control			+++++	+++++	153	69
Formaldehyde	1	0.025	++	+++++	148	65
	1	0.05	+	+++++	144	65
	1	0.10	−	+++	138	60
	6	0.025	++	+++++	145	65
	6	0.05	+	+++	139	60
	6	0.10	−	+++	127	56
8-hydroxy-quinoline sulfate	1	0.025	++	+++	137	57
	1	0.05	−	+++	125	50
	1	0.10	−	+++	113	40
	6	0.025	+	+++	120	53
	6	0.05	−	+++	107	39
	6	0.10	−	++	78	34
Mercuric chloride	1	0.05	−	+++		
	1	0.10	−	++		
Methyl mercuri-cyandiamide (Panogen)	1	0.025	+	+++++		
	1	0.05	−	+++++		
	1	0.10	−	+++		
o-Phenyl-phenate (Dowicide A)	1	0.05	−	+++		
	1	0.10	−	+++		
	1	0.15	−	+		

a Five plus signs indicate mold growth equivalent to that of the control. Fewer plus signs indicate progressively less mold growth and a minus sign indicates the absence of mold.
b Five plus signs indicate acrospire development equivalent to that of the control. Fewer plus signs indicate progressively less acrospire growth.
c See ref. 21.
d See ref. 16.

effects of formaldehyde, 8-hydroxyquinoline sulfate, *o*-phenylphenate, methylmercuricyandiamide, and mercuric chloride on acrospire growth and enzyme development during malting are given in Table II. The 0.10% concentration of formaldehyde in steepwater during the final 1- and 6-hour periods completely prevented growth of fungi. The 0.05% concentration was almost completely effective, while 0.025% caused a pronounced reduction in mold growth. The formaldehyde treatments caused moderate reductions in enzyme activities. This effect may be countered by including gibberellic acid in the steepwater. Similar effects were reported by Grace (10), who indicated that the reduction in germination caused by formaldehyde could be reversed by 1-naphthylacetic acid or 3-indoleacetic acid.

At concentrations of 0.05% or more 8-hydroxyquinoline sulfate completely inhibited fungi, and it gave very good results at 0.025% concentration. This substance, however, severely reduced enzyme activities and may be of little importance from a practical standpoint because of its possible toxicity to animals and man (8).

Mercuric chloride, *o*-phenylphenate (Dowicide A),[3] and methyl mercuricyandiamide (Panogen) at concentrations of 0.05% or more of the active chemical completely controlled growth of fungi when present in the steepwater during the final hour of the steeping. Mercuric chloride and Dowicide A, however, materially reduced seed germination. Panogen, at concentrations of 0.025 to 0.1%, had little effect on acrospire growth. These results suggest that damp wheat is subject to damage by most mercurial and phenolic fungicides, and confirm observations of Roane and Starling (20) concerning the mercurial compounds. These materials would seem to be of no practical importance in malting, because of their highly toxic nature. For this reason data concerning their effect on enzyme activity were not determined.

Figure 1 illustrates the effect of formaldehyde, calcium and sodium hypochlorite, sorbic acid, potassium sorbate, and 8-hydroxyquinoline sulfate on the growth of molds during wheat germination. Treatment with either formaldehyde or 8-hydroxyquinoline sulfate was almost completely effective at the 0.025 and 0.05% levels. The hypochlorites, potassium sorbate, and sorbic acid had little or no effect when used at comparable concentrations. Treatment with 8-hydroxyquinoline sulfate reduced rootlet and shoot growth appreciably.

Conditions optimum for the development of fungi are not necessarily so for the development of the acrospire when the agar plate

[3] The mention in this publication of a trade product does not imply its endorsement by the U.S. Department of Agriculture over similar products not named.



Fig. 1. Effect of chemical treatments on the growth of fungi on wheat during malting. First row, L to R: Control, 0.025% formaldehyde, 0.050% formaldehyde. Second row, 0.10% calcium hypochlorite, 0.04% sorbic acid, 0.07% sorbic acid. Third row, 0.10% sodium hypochlorite, 0.2% potassium sorbate, 0.3% potassium sorbate. Fourth row, 0.10% 8-hydroxyquinoline sulfate, 0.05% 8-hydroxyquinoline sulfate, 0.025% 8-hydroxyquinoline sulfate.

technique is used. Hence, data for Fig. 1 concerning acrospire length may not agree with data in Tables I and II which were obtained by malting.

Residual Formaldehyde in the Malt. The results of assays of formaldehyde-treated malts for residual extractable formaldehyde are given in Table III. Amounts found in the finished malt were as little as 0.003 p.p.m. The study of the effect of formaldehyde on yeast fermentation which made use of pressuremeters (1) indicated that 10

TABLE III

FORMALDEHYDE REMAINING IN STEEPWATER AND IN FINISHED MALT[a]

ADDED TO STEEPWATER	REMAINING IN STEEPWATER	FOUND IN WHEAT AFTER STEEPING	FOUND IN MALT AFTER KILNING
mg	*mg*	*mg*	*mg*
Formaldehyde present for final hour of steep period			
49.2	47.4	2.0	0.07
25.2	24.1	1.2	0.05
11.9	10.7	1.3	0.03
Formaldehyde present for final 6 hours of steep period			
49.2	46.3	3.0	0.08
25.2	23.2	2.1	0.05
11.9	10.3	1.6	0.05

[a] Fifty grams of Triumph wheat steeped in 50 ml. of water with formaldehyde present for 1 or 6 hours.

or fewer p.p.m. of formaldehyde in doughs had no effect on gas production. Because the amount of malt which is added customarily to bread doughs is but 0.25–0.75%, based on flour weight, the amount of added formaldehyde would have no effect on gas production. The residual formaldehyde in bread, if 0.5% treated malt was used, would be 10–5 p.p.m. Ng, Reed, and Pence (17) have extracted formaldehyde from fresh bread. Presumably the formaldehyde is produced during yeast fermentation, since the authors have found formaldehyde in the gases evolved from pre-ferments.

High moisture content of germinating grain and the high humidity maintained during malting make possible the dissipation of formaldehyde rather than its conservation through polymerization to paraformaldehyde.

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THE BROMATE REACTION IN DOUGH
III. Effect of Continuous Mixing and Flour Particle Size[1]

W. BUSHUK AND I. HLYNKA

ABSTRACT

The bromate reaction in a flour-water dough which is continuously mixed under nitrogen can be characterized by a single continuous reaction curve in contrast to the apparent two-phased reaction curve obtained under normal conditions comprising 5 minutes of mixing followed by storage of the dough under controlled conditions. More bromate reacts in the continuously mixed dough than in the normal dough. In the range of potassium bromate concentrations from 0 to 37.50 mg. per kg. of dough (0 to 60 p.p.m. of flour), the reaction in the mixed dough is apparently first-order but the specific rate constant decreases with increasing bromate concentration. At a concentration of 18.75 mg. per kg., the equation for the specific rate constant is

$$k = 1.08 \times 10^{4} e^{-11,600/RT} \text{ sec}^{-1}.$$

In the normal dough, the rate of the so-called linear reaction seems to be partially controlled by a physical process. This rate-controlling factor seems to enter into the kinetics of the reaction primarily through the entropy of activation and seems to be related to the accessibility of sulfhydryl groups. The accessibility seems to depend on the specific surface of the flour particles.

This paper, the third of the present series (1,2), compares results of a study of the reaction of the bromate ion in normal flour-water

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doughs with that in doughs which are mixed continuously throughout reaction periods of up to 5 hours, and attempts to explain the differences found in terms of the energy and entropy of activation. It also presents results on the effect of flour particle size which was investigated because it offered possible experimental verification of the hypothesis that continuous mixing increases the physical accessibility of sulfhydryl groups. It is desirable to report the results at this stage of the investigation of the bromate reaction in dough, as they seem pertinent to the setting-up of the mechanism for the reaction as well as having certain practical implications.

Materials and Methods

The flour used for the continuous mixing study was the straight-grade flour described previously (1). This flour has now been in cold storage for one and a half years and consequently the rate of the bromate reaction in a normal, control dough has decreased by about 15% (3).

The flour samples used to study the effect of particle size were specially milled in the Laboratory from a high-grade Canadian hard red spring wheat. The wheat was milled into farina which passed through sieve 30GG and was held on 72GG. A portion of this farina was then reduced to a size which passed through 72GG and was held on 10xx and a second portion was reduced to pass through 10xx (flour). A portion of the flour was further reduced by ball-milling for 16 hours. The protein and ash contents of these flours are given in Table I. The increase in the ash content during ball-milling is attributed to small fragments of ball-mill stones produced during ball-milling. These ash-producing components seem to be inert toward the bromate reaction.

TABLE I
DESCRIPTION OF THE FLOURS OF DIFFERENT PARTICLE SIZE

	Particle Size	Protein Content	Ash Content
		%(14% m.b.)	*%(14% m.b.)*
Farina	Thru 30GG; over 72GG	11.7	0.382
Reduced farina	Thru 72GG; over 10xx	11.7	0.381
Flour	Through 10xx	11.8	0.381
Ball-milled flour		11.6	0.501

The method of analysis for residual bromate ion in dough was described previously (1). In the continuous mixing study, doughs of 60% absorption were mixed under a slightly positive pressure of nitrogen in the GRL mixer (5) throughout reaction periods of up to 5

hours. A change in the mixing rate to 2.57 times the standard rate of 75 r.p.m. of outside pins was obtained by adjustable V-pulleys. To eliminate possible contamination with atmospheric oxygen, a separate dough was mixed for each reaction time. To control the dough temperature at 30°C., the temperature of the circulating water had to be maintained at 29°C. to overcome the heat produced by the mixing. The temperature of the dough was measured with a mercury thermometer immediately after mixing.

In the discussion that follows, the type of dough that was mixed for 5 minutes and then stored under controlled humidity and temperature during the reaction time will be referred to as a normal dough; the other type of dough used in this study will be referred to as a continuously mixed dough.

Results and Discussion

Effect of Continuous Mixing on the Reaction. Figure 1 compares the bromate reaction in a normal dough and in doughs mixed continuously at two different mixing rates for a potassium bromate concentration of 18.75 mg. per kg. of dough. The amount of bromate ion that reacts in a continuously mixed dough is about 50% higher than that in a normal dough. Furthermore, the reaction in the continuously mixed dough can be represented by a smooth curve, whereas in the normal dough the reaction seems to be two-phased over a fairly wide range of experimental conditions (1,2). Similar differences were

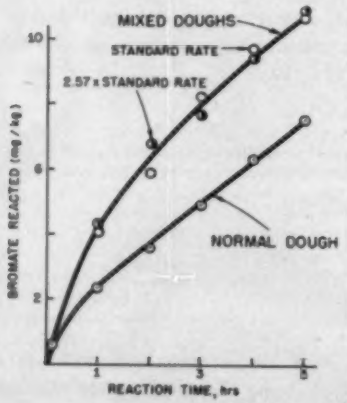


Fig. 1. Reaction of the bromate ion in normal and continuously mixed doughs for initial potassium bromate concentration of 18.75 mg. per kg. of dough.

found at other bromate levels in the range 6.25 to 37.50 mg. per kg. that were investigated.

It can be generalized from the results of Fig. 1 that the linear character of the secondary phase of the bromate reaction in the normal dough arises from the fact that some physical process such as diffusion becomes a major rate-controlling factor when the dough is not being mixed. It seems that this factor can be eliminated almost completely by continuous mixing at the standard rate used in this Laboratory, since no further increase in the bromate reaction was observed when the continuous mixing rate was increased to 2.57 times the standard rate.

In the two subsections that follow, certain features of the effect of continuous mixing will be examined in terms of kinetic and thermodynamic parameters that can be derived from primary data of the type described above. Other aspects of the physical rate-controlling factor will be discussed below when additional experimental data have been presented.

Kinetic Order of the Reaction. Figure 2 gives first-order plots for the data of Fig. 1. In these plots the logarithm of the residual bromate concentration is plotted as a function of reaction time. The results for the high mixing rate are not included in these plots. Both reactions appear to be first order, although the plot for the reaction in the normal dough deviates from linearity when a more expanded ordinate scale is used, and a definite change in slope for the initial and the linear phases is indicated.

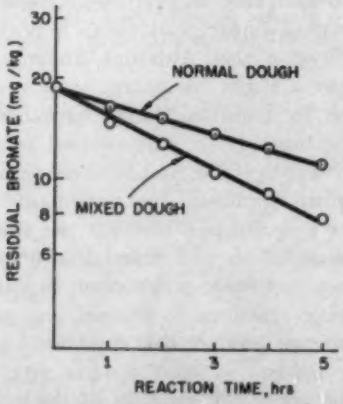


Fig. 2. First-order plots for the reaction of bromate ion in normal and continuously mixed doughs for initial potassium bromate concentration of 18.75 mg. per kg. of dough.

To examine further the first-order character of the bromate reaction in a continuously mixed dough, reaction curves were obtained for a series of potassium bromate concentrations in the range 6.25 to 37.50 mg. per kg. Although the first-order plots of the type shown in Fig. 2 were reasonably linear for all the concentrations used, the specific rate constant decreased slightly with increasing concentrations (see Table II). Accordingly, it seems that the bromate reaction in a continuously mixed dough is not a true first-order reaction and perhaps it might be more correct to refer to it as a *pseudo* first-order reaction.

TABLE II
SPECIFIC RATE CONSTANTS AT VARIOUS POTASSIUM BROMATE CONCENTRATIONS

POTASSIUM BROMATE CONCENTRATION		SPECIFIC RATE CONSTANT $\times 10^5$
mg/kg dough	*ppm flour*	sec^{-1}
6.25	10	6.47
12.50	20	5.52
18.75	30	4.87
25.00	40	4.45
37.50	60	4.00

It should be emphasized that reference to bromate reaction in either type of dough as being first-order does not imply that the reaction is unimolecular. The combination of the various rate-controlling factors apparently gives a reaction such that when the logarithm of the residual bromate ion concentration is plotted against reaction time a straight line results. This property is sufficient to classify the reaction as having an over-all kinetic order of one. Classification of the so-called linear reaction in a normal dough as first-order needs amplification also. Although under some conditions the reaction curve shows a slight curvature, good straight lines for this part of the reaction in a normal dough have been obtained over a wide range of conditions (1,2). The over-all reaction in a normal dough was divided into a rapid, initial reaction and a slower linear reaction, primarily to emphasize the two-phased nature of the reaction. However, it is quite possible that the linear reaction represents a small segment of an extremely slow first-order reaction.

Activation Energy and Frequency Factor. Figure 3 gives Arrhenius plots for the bromate reactions in normal and continuously mixed doughs for a potassium bromate concentration of 18.75 mg. per kg. The slopes by the method of least squares give 11,600 and 10,160 cal. per mole as the activation energies of the reactions in continuously mixed and normal doughs respectively. The frequency factors, determined from the intercepts of the lines in Fig. 3, are 1.08×10^4

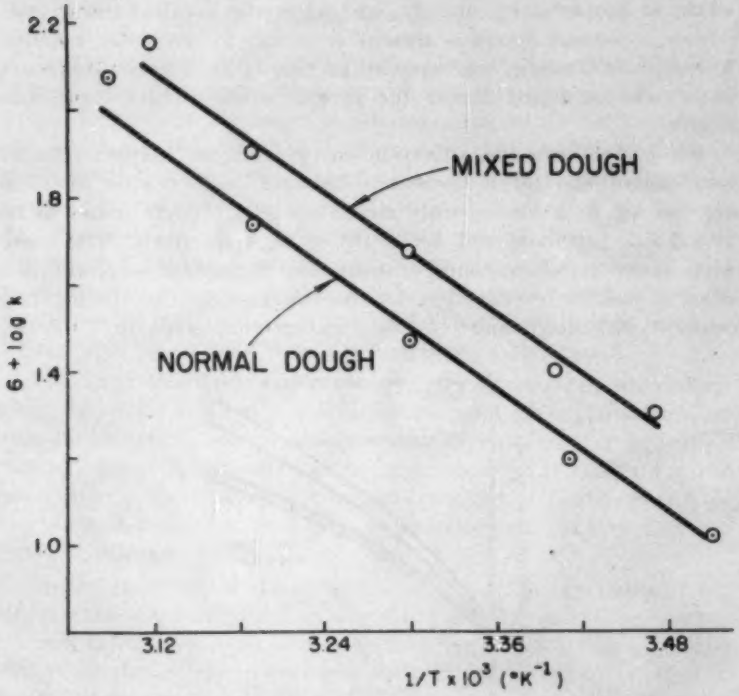


Fig. 3. Arrhenius plots showing the logarithm of the rate constant as a function of the reciprocal of the absolute temperature for bromate reactions in normal and continuously mixed doughs for an initial potassium bromate concentration of 18.75 mg. per kg. of dough.

sec^{-1} and 0.80×10^4 sec^{-1} for the reactions in continuously mixed and normal doughs.

If the above values are substituted into the integrated form of the Arrhenius equation

$$k = A e^{-E/RT} \tag{1}$$

where A is the frequency factor and E the activation energy, the rate constants for the concentration of potassium bromate used take on the values:

$$k = 1.08 \times 10^4 e^{-11,600/RT} sec^{-1} \tag{2}$$

and

$$k = 0.80 \times 10^4 e^{-10,610/RT} sec^{-1} \tag{3}$$

for the reactions in continuously mixed and normal doughs respectively. The same activation energy and frequency factor (con-

verted to proper units) are obtained when the so-called linear reaction in a normal dough is treated according to zero-order kinetics. The activation energy was reported previously (1) and the frequency factor was calculated during the present study using the previous results.

For comparison, the activation energy and the frequency factor were determined for a potassium bromate concentration of 12.50 mg. per kg. in a continuously mixed dough and were found to be 10,800 cal. per mole and 1.07×10^4 sec^{-1}, respectively. The somewhat lower activation energy at the lower bromate concentration offers a possible explanation for the increase in the specific rate constant with decreasing bromate concentration given in Table II.

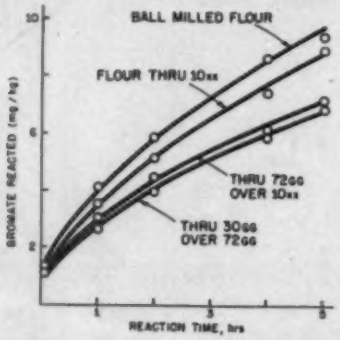


Fig. 4. The bromate reaction at 30°C. in normal doughs prepared from flours of different particle size.

It is possible that the extent to which the physical accessibility factor controls the rate of reaction varies with bromate concentration.

Effect of Flour Particle Size. Figure 4 shows the bromate reaction in normal doughs mixed under nitrogen to 60% absorption from flours which differed only in the size of the particles comprising each sample, with the exception of the ball-milled flour which also had a higher ash content. The initial potassium bromate concentration in this study was 18.75 mg. per kg. of dough. It is seen that the rate of bromate disappearance increases with increasing specific surface (decreasing particle size). Since this Laboratory does not have facilities for measuring flour particle size, it was not possible to determine the exact relationship between the rate of bromate reaction and the specific surface area. It is anticipated that establishment of such a relationship would be complicated by a particle heterogeneity factor which might be different for different particle size fractions.

General Discussion

An attempt will first be made to explain the observed difference between the bromate reactions in continuously mixed and normal doughs in terms of the theory of rate processes (4). In normal doughs the initial reaction, which is analogous to the reaction in continuously mixed doughs, gives place to what appears to be a linear reaction. The most probable explanation is that some physical factor, not necessarily wholly inoperative during mixing, becomes rate-controlling in a resting normal dough. Both the initial reaction in a normal dough and the reaction in a continuously mixed dough are quantitatively described by equation 2 whereas the linear phase of the reaction in a normal dough is described by equation 3.

The large difference in the rates of the reaction in the doughs subjected to the two types of treatment cannot be explained on the basis of the small, almost insignificant difference in the activation energies. Nevertheless, the slightly lower activation energy for the reaction in the normal dough suggests that the activation energy of the physical process is probably somewhat lower than that of the chemical reaction.

On the other hand, the difference in the frequency factor is significant and parallels quite closely the difference in the amount of reaction in the two types of doughs. The frequency factor is directly related to the entropy of activation (4), and it therefore can be concluded that the physical rate-controlling factor affects this thermodynamic parameter. It is postulated that the increase in the entropy of activation as a result of continuous mixing occurs because reaction sites which may otherwise be sterically inaccessible are uncovered and increase the probability of effective collisions.

If the physical accessibility postulate is correct, then it might be possible to substantiate it experimentally by determining the rate of bromate disappearance in doughs prepared from flours of different particle size. Results of Fig. 4 showed that the rate increases as the particle size of the flour decreases. Since it is difficult to presume that reduction of a coarse farina to flour would produce new sulfhydryls by physically rupturing disulfide linkages, the increase in the bromate reaction must be due to an increase in the accessibility of the sulfhydryl groups with which the bromate ion seems to react. It should be emphasized that demonstration of the physical accessibility factor in the bromate reaction is possible only because the rate of the chemical reaction is of the same order of magnitude as the rate of the physical process. The evidence that the effect of con-

tinuous mixing is analogous to an increase in specific surface indicates that flour particles, mixed into a dough, retain a certain amount of the original identity, which probably decreases during prolonged mixing owing to a continuous shearing-off of the gluten layer formed on the external surface of the flour particle during dough development. The possibility that flour particles retain their identity in dough offers an interesting avenue of research on the physical properties of dough.

In conclusion, it should be pointed out that although the findings reported in this paper appear to cast some doubt on the usefulness of the results obtained with normal doughs for elucidating the mechanism of the bromate reaction, such results are necessary for our understanding of the reaction that occurs under a range of conditions used in baking technology. The effects of continuous mixing and flour particle size seem to complicate the kinetics slightly; however, it will be the understanding of all aspects of the bromate reaction in dough that will ultimately yield the mechanism of the improver reaction. The results of the continuous mixing experiments do indicate that the bromate reaction in doughs handled according to the more traditional baking practice is probably different from that in doughs produced by the high-speed mixing and mechanical development employed in modern baking technology. The results obtained with flours of different particle size indicate that special precautions must be observed in studying bromated doughs from flours prepared by the air-classification process.

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ZONE ELECTROPHORESIS PATTERN OF FREE AMINO ACIDS AS AN INDEX OF STORAGE CONDITION OF WHEAT[1]

PEKKA LINKO[2]

ABSTRACT

Paper electrophoresis of free amino acids provided a rapid, simple, and convenient method to detect changes in the free amino acids of wheat during storage. Using 0.025*M* potassium-hydrogen-phthalate solution (pH 4.0) as buffer, a good resolution of aspartic, glutamic, gamma-aminobutyric, and neutral amino acids (as a group), as well as arginine and lysine, was obtained with 400 volts (14.0 V/cm.) during 90 minutes. For practical purposes 500 volts (18.5 V/cm.) and 30 minutes gave a satisfactory separation, yet no cooling was necessary.

The electrophoresis patterns of free amino acids of wheats of good quality and high viability were characterized by a high glutamic acid peak, an average ratio of glutamic acid/aspartic acid peak height over 2, and a negligible gamma-aminobutyric acid peak. Glutamic acid peak and glutamic acid/aspartic acid peak height ratio decreased as deterioration increased. A marked gamma-aminobutyric acid peak was present without exception in electrophoresis patterns of wheats stored at unfavorable conditions. Dead wheats of extensive deterioration exhibited an increase in most of the free amino acids, including glutamic acid, largely due to protein breakdown.

Glutamic acid peak increased markedly in germinated wheats, with moderate increases in aspartic and basic amino acids and little increase in gamma-aminobutyric acid.

Although the total quantity of free amino acids in stored wheat has been reported to be affected little during the early stages of deterioration (10), Jones and Gersdorff (4) observed that prolonged storage of wheat and milled wheat products decreases protein nitrogen and increases free amino acid nitrogen. DeVay (3) observed changes in the concentrations of certain individual free amino acids in hard red spring wheat (Lee variety) during storage at 19.5% moisture. The most notable change was the appearance of gamma-aminobutyric acid in moldy moist wheat. Linko and Milner (7) showed recently that a considerable change in the composition of the free amino acids of wheat may take place immediately following wetting. Linko (5) studied the effect of storage at 16.2% moisture at 37°C. on the individual free amino acids of Seneca wheat, analyzing the embryo and the endosperm end separately. The most significant change during the first two days of storage was the almost total and irreversible loss of free glutamic acid of the embryo, accompanied

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by as striking an increase in free gamma-aminobutyric acid, evidently due to the activation of glutamic acid decarboxylase by the increased moisture content (1,8). Except for arginine, glutamic acid, and the amides, free amino acids in the endosperm end of the kernel generally increased as a result of proteolysis.

Hence, though the total quantity of free amino acids exhibits a significant increase only at advanced stages of deterioration, it was thought that methods to detect changes in individual amino acids could prove valuable in estimating the degree of deterioration. Paper chromatography of free amino acids showed some promise in this respect (6), but it is relatively time-consuming. Paper electrophoresis with high voltage gradients would not have that disadvantage. Hence it appeared to provide a simple technique to estimate the storage condition of wheat.

Materials and Methods

Fifty-two wheat samples, including several varieties of high viability from 1956 to 1959 crops and some commercial wheats at various stages of deterioration, were investigated. All were stored in moisture-proof containers at 4°C. until the analyses were made. In addition, samples of Pawnee wheat were conditioned to various moisture levels and stored 1 year at 25°C. To compare the changes in free amino acids during storage with those taking place during the early stages of germination, such as may be encountered in sprout-damaged wheats, Seneca wheat was steeped 16 hours at 25°C., followed by 40 hours of germination between filter papers. Samples were taken at suitable time intervals and dried in a forced-air oven at 38°C. for 24 hours.

Extraction of Free Amino Acids. Wheat was ground 2 minutes with a Waring Blendor and mixed well. Five grams (dry weight) were extracted in a mortar with approximately 10 ml. of 70% (w/v) ethanol, followed by centrifugation. This procedure was repeated five times, after which the combined ethanol extracts were passed through an Amberlite IR-120 (H^+) resin column (1 ml. per minute; column 6×100 mm.; resin 16 to 50 mesh, U.S. Standard screen). The column was washed with 25 ml. of 70% (w/v) ethanol, after which the amino acids were displaced by 25 ml. of 1*N* ammonium hydroxide. The eluate was vacuum-distilled to 1 ml. (bath temperature 60°C.).

Electrophoresis. A Durum type Spinco paper electrophoresis apparatus, Model R, Series B, was used. A 20-μl. aliquot, corresponding to 100 mg. (dry weight) of wheat, was pipetted as a narrow streak

(c. 2 × 25 mm.) on the center of the 30-mm.-wide filter paper strip (precut Spinco filter paper, No. 300–028). From several buffers tried, 0.025*M* potassium-hydrogen-phthalate (pH 4.0) proved most suitable. Electrophoresis was continued for 90 minutes at 400 V (14.0 V per cm.). The strips were dried horizontally in a forced-air oven at 60°C., dipped in 0.25% ninhydrin in acetone, heated 2 minutes at 80°C., left standing at room temperature overnight, and finally dipped for a moment in a cupric nitrate solution (2 ml. of saturated cupric nitrate in water and 0.2 ml. of 10% nitric acid, filled to 100 ml. with acetone). After drying for about 20 minutes at room temperature the strips were automatically scanned with the Spinco Analytrol using 1.5-mm. slit and 500-mμ filter.

Results and Discussion

Figure 1 shows that, using 0.025*M* potassium-hydrogen-phthalate (pH 4.0) as buffer, aspartic acid, glutamic acid, alanine (and other neutral amino acids as a group), gamma-aminobutyric acid, arginine, and lysine were separated in 90 minutes with 400 V (14.0 V per cm.). Beta-alanine would appear between the neutral amino acids and

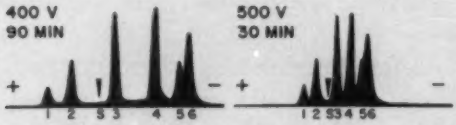


Fig. 1. Paper electrophoresis patterns of authentic amino acids in 0.025*M* potassium-hydrogen-phthalate (pH 4.0). S, point of application; 1, aspartic acid; 2, glutamic acid; 3, alanine + glycine (representing the neutral group); 4, gamma-aminobutyric acid and 5, arginine; 6, lysine.

gamma-aminobutyric acid, but it was omitted from the figure because it was found only in trace amounts in wheat. Figure 1 shows, in addition, that for most practical purposes 30 minutes' electrophoresis with 500 V (18.5 V per cm.) satisfactorily resolves these amino acids. The partial overlapping of arginine and lysine peaks is not of great importance because of the low lysine concentration in wheat. Phosphate buffer (0.067*M*, pH 7.0; 300 V and 6 hours) was not satisfactory, because it did not resolve aspartic acid from glutamic acid, nor gamma-aminobutyric acid from the group of neutral amino acids. Furthermore, evaporation due to heating was undesirably great. Hence 0.025*M* phthalate buffer, 400 V, and 90 minutes were adopted as standard conditions because of a good resolution in a relatively short time, without requiring cooling.

Figure 2 shows examples of electrophoresis patterns for Pawnee wheat stored at various moisture levels at 25°C. for 1 year. Table I shows the viability and mold count (2) of these samples after storage. The shoot length of germinated (for 4 days) kernels stored at 14.5% original moisture content was slightly less than one-half of that of wheat stored at 11.0% original moisture level. All samples stored at

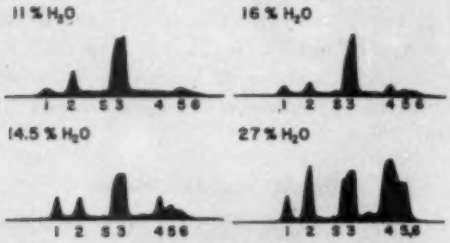


Fig. 2. Paper electrophoresis patterns of free amino acids of Pawnee wheat stored at various moisture levels at 25°C. for 1 year. See Fig. 1 for key to abbreviations.

TABLE I

VIABILITY AND MOLD COUNT OF PAWNEE WHEAT STORED AT VARIOUS MOISTURE LEVELS AT 25°C. FOR ONE YEAR

Moisture		Germination	Mold Count
Before Storage	After Storage		
%	%	%	colonies/g
11.0	9.2	96	7,000
14.5	11.7	41	9,700
16.0	12.4	0	200,000
18.9	24.2	0	>10,000,000
27.0	33.2	0	52,000
36.0	40.0	0	10,500

higher moisture levels had totally lost viability. At original moisture levels below 16.0% the mold count remained relatively constant, which agrees well with the findings of Milner, Christensen, and Geddes (9). The sample conditioned to 16.0% moisture molded very slowly, whereas mold growth was excessive from the very beginning in the sample of 18.9% moisture. In the two high-moisture samples vigorous mold growth was observed in a few days. Later, in about a week, it receded and molds vanished owing to excessive carbon dioxide evolution from the grain. No mold growth could be visually detected in these samples during the rest of the experimental period.

Despite the small increase in mold count, gamma-aminobutyric acid peak was clearly noticeable in the sample with 14.5% original moisture. At 16.0% moisture level the glutamic acid peak had mark-

edly decreased in size, owing to the activation of glutamic acid decarboxylase (8). At 27% moisture level extensive proteolysis had taken place resulting in the production of large quantities of glutamic acid, followed by some decarboxylation to gamma-aminobutyric acid. Hence, if wheat has been stored under very unfavorable conditions the glutamic acid peak may be excessive, but it is then always accompanied by a high gamma-aminobutyric acid peak; this distinguishes the pattern from that of wheat of high quality with a high glutamic acid peak but a negligible gamma-aminobutyric acid peak.

Generally, the patterns presented in Fig. 2 seemed to be characteristic for wheats of different variety and of different condition. This was evident from results presented in Figs. 3 and 4. A relatively high glutamic acid peak, with low ones for aspartic and basic amino acids and virtually no detectable gamma-aminobutyric acid peak, is significant for good-quality wheats of high germination percentage (Fig. 3). The peak height ratio glutamic acid/aspartic acid in these wheats averaged 2.8, and it decreased with increasing deterioration

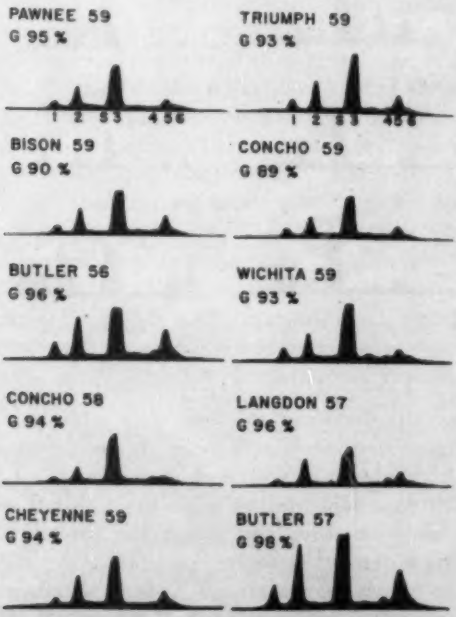


Fig. 3. Paper electrophoresis patterns of free amino acids of wheats of high viability, stored at 4°C. See Fig. 1 for key to abbreviations.

to below 1.0 as can be seen from Fig. 4. Slight variations in the area of the neutral amino acid peak were not significant, since the total quantity of the free amino acids varies somewhat in different wheat samples and since the high total concentration of the neutral amino acids does not permit an accurate determination with the technique. Figure 4 shows electrophoresis patterns of free amino acids obtained from various commercial wheats. When viability decreases, glutamic

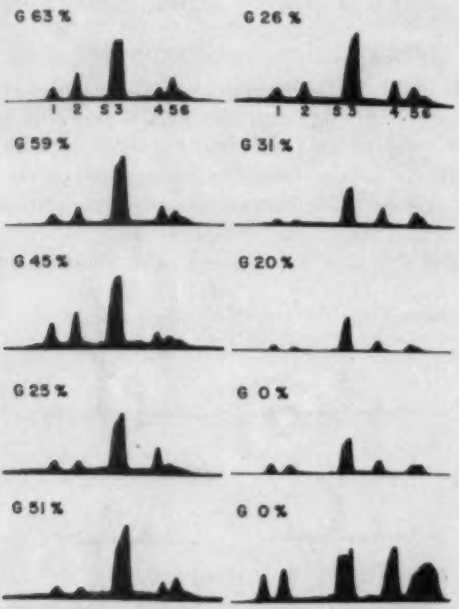


Fig. 4. Paper electrophoresis patterns of free amino acids of different lots of commercial wheats at various stages of deterioration. See Fig. 1 for key to abbreviations.

acid peak generally decreases, followed invariably by a more or less marked gamma-aminobutyric acid peak. It should be noticed, however, that if high glutamic acid peak is accompanied both by high gamma-aminobutyric acid and by high basic amino acid peaks, deterioration is very extensive and wheat has probably been dead a long time under unfavorable storage conditions.

As shown in Fig. 5, the glutamic acid peak also increases markedly during the early stages of germination, followed by a lesser increase in aspartic acid and, later, by increases in basic amino acids. That

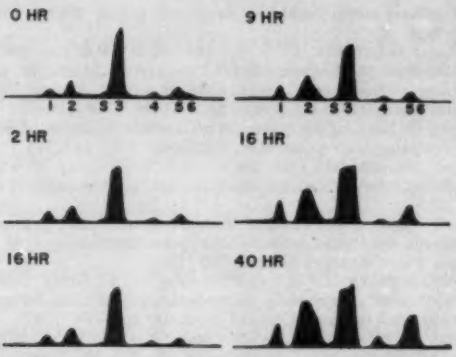


Fig. 5. Paper electrophoresis patterns of free amino acids of Seneca wheat, steeped 0, 2, and 16 hours at 25°C. (left), and steeped 16 hours followed by 9, 16, and 40 hours of germination between filter papers at 25°C. (right). See Fig. 1 for key to abbreviations.

no marked increase in gamma-aminobutyric acid peak could be noticed during the first 2 days of germination distinguished the patterns of sprouted wheats from those of advanced storage deterioration.

Although the method at its present state does not accurately determine the germination percentage in a given sample of wheat, the general pattern of the free amino acids obtained by the technique of paper electrophoresis promises to provide a simple estimation of the average storage condition. The results agree well with the earlier observations of Linko (5) concerning changes in the composition of the free amino acids during storage and germination of wheat. Such patterns as presented in Fig. 3, with a relatively high glutamic acid peak, a glutamic acid/aspartic acid peak height ratio over 2, and a small gamma-aminobutyric acid peak, would indicate that wheat has been stored under good conditions, the germination being most likely between 80 and 100%. Appearance of a gamma-aminobutyric acid peak, accompanied by a relative decrease in the glutamic acid peak, invariably indicates a decrease in quality. This type of pattern would also reveal damage caused by temporarily elevated moisture content during the storage period, regardless of the present moisture level of the grain examined.

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A NOTE ON SELF-DIFFUSION IN DOUGH CONTAINING Br^{82}-LABELED BROMATE[1]

C. C. LEE[2] AND R. TKACHUK[3]

Diffusion of mass refers to the dissipation of a concentration gradient by molecular transfer with no over-all mass flow caused by external forces. Self-diffusion differs from ordinary diffusion in that its occurrence does not require the existence of an over-all chemical concentration gradient. The method used for studies of self-diffusion generally involves the observation of the net translational motion of a given amount of substance labeled with a known isotope of one of the constituent elements in an otherwise chemically uniform medium. The self-diffusion rate thus gives the nearest available approach to the observation of the magnitude of actual molecular motion in the gross sense (6). As an attempt to obtain some indications of the gross mobility of bromate and its decomposition product, bromide, in doughs, a study on self-diffusion was made with dough initially containing 30 p.p.m. potassium bromate.

Two pieces of dough were made up with 100 g. of flour and 60

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[3]Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg 2, Manitoba.

ml. of distilled water by mixing for 3 minutes in a Hobart mixer. One contained 30 p.p.m. ordinary potassium bromate and the other contained the same amount of Br^{82}-labeled potassium bromate (7) with initial specific activity of about 5×10^5 c.p.m. per mg. The doughs were joined together, being separated only by a moist sheet of Whatman No. 4 filter paper which made possible a clean division at the boundary at the end of the diffusion period. Diffusion was allowed to proceed for 4 hours and 20 minutes in a cabinet maintained at 30°C. and 80% relative humidity. The doughs were then frozen and separated at the boundary. Cores of dough, 23 mm. in diameter, were cut at right angles to the boundary from each of the two pieces and then sliced into samples of approximately 2-mm. thickness. The radioactivity of each sample was measured and the actual sample thickness was estimated from the sample weight with the assumption that dough density was 1.2. Duplicate experiments were carried out and the results are given in Table I.

TABLE I

Br^{82} Activity from Self-Diffusion in Dough

Sample[a]	Net Activity		Sample Thickness		Cumulative Distance from Boundary	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
	cpm	cpm	mm	mm	mm	mm
1a	74.6	87.6	2.0	2.7	2.0	2.7
2a	95.4	99.8	1.8	0.9	3.8	3.6
3a	112.2	116.2	2.1	1.8	5.9	5.4
4a	119.9	113.1	2.0	1.4	7.9	6.8
5a	121.8	125.7	2.2	1.8	10.1	8.6
6a		119.8		2.0		35
1b	36.1	37.0	1.8	1.4	1.8	1.4
2b	21.7	19.7	1.9	1.7	3.7	3.1
3b	10.2	7.3	2.3	1.5	6.0	4.6
4b	0.9	1.7	2.3	2.6	8.3	7.2
5b	0	0	2.3	1.6	10.6	8.8
6b		0.1		2.2		35

[a] The "b" samples were derived from dough which initially contained nonradioactive bromate.

When sample activities were plotted against distance from the boundary, typical diffusion curves were obtained. The self-diffusion coefficient, D, was calculated from a solution of Fick's law given by Barrer (1) for the case of diffusion across a sharp boundary in an "infinite solid" where no appreciable amount of material diffuses from the far extremities of the system. The solution (1) takes the form of equation [1], where C_o is the initial concentration, C is the concentration at distance x, and t is the time. The second term of equation [1] is the Gaussian error function; hence [1] may be written as equation [2].

$$C = \frac{C_o}{2}\left[1 + \sqrt{\frac{2}{\pi}}\int_0^{\frac{x}{2\sqrt{Dt}}} e^{-y^2} dy\right] \qquad [1]$$

or

$$C = \frac{C_o}{2}[1 + \text{erf}(y)] \qquad [2]$$

Using the curves derived from the two sets of experimental data given in Table I, an average activity value, C, corresponding to a given distance from the boundary, x, can be obtained graphically. With these values of C and x, equations [1] and [2], and mathematical tables of error functions (4,5), the diffusion coefficient D can be evaluated. In this way, a number of D values corresponding to various distances x were calculated, giving rise to a mean value of 0.24 cm.^{2} day^{-1} for D.

As a check on the mean value of 0.24 cm.^{2} day^{-1} as a valid self-diffusion coefficient for the present data, the theoretical curve for this D value was calculated using equation [3] derived from expanding equation [1] as a series (1).

$$C = \frac{C_o}{2}\left[1 \pm \frac{2}{\sqrt{\pi}}\left\{\frac{x}{2\sqrt{Dt}} - \frac{x^3}{3 \cdot 1!\,(2\sqrt{Dt})^3} + \frac{x^5}{5 \cdot 2!\,(2\sqrt{Dt})^5} - \frac{x^7}{7 \cdot 3!\,(2\sqrt{Dt})^7} + \cdots\right\}\right] \qquad [3]$$

This theoretical curve is shown in Fig. 1, together with the data from Table I being given as observed experimental points. It is evident from Fig. 1 that in the water-flour dough system, the move-

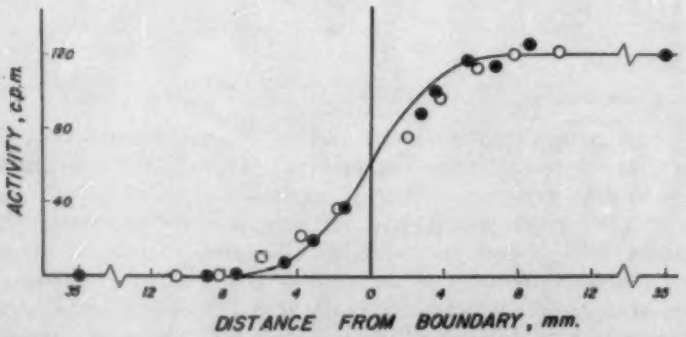


Fig. 1. Self-diffusion of Br^{82} in dough. The theoretical curve for D equals 0.24 cm.^{2}day^{-1} is shown together with data from Table I as observed experimental points. Open circles are data from trial 1; solid circles from trial 2.

ment of Br^{82}-labeled ions, which would include both undecomposed bromate and the decomposition product, bromide, follows the laws of diffusion and fits reasonably well with a self-diffusion coefficient of 0.24 cm.^{2} day^{-1}.

The self-diffusion coefficients of bromate and bromide in the anion exchanger, Dowex-2, have been found to be, respectively, 4.55 and 3.87×10^{-7} cm.^{2} sec.$^{-1}$ at 25°C. (8). The self-diffusion coefficient of bromide in 0.05 molar sodium chloride at 25°C. was reported as 1.62 cm.^{2} day^{-1} (2). In cereal chemistry, from the properties of the resulting doughs when fermented under carbon dioxide, oxygen, or nitrogen, Sullivan and Richards (9) have postulated that the sponge is permeable to the gas above it. In an attempt to substantiate this postulate, Burtle and Sullivan (3) were able to show that radioactive carbon dioxide can diffuse back into the sponge even against the developing internal pressure of the dough. The present study on self-diffusion clearly demonstrated that, under the conditions used in these experiments, net movements of bromate and bromide in the dough are detectable within about 1 cm. on either side of the boundary. This finding points to a dynamic nature for the soluble constituents of dough which may merit further investigation.

Acknowledgments

The authors wish to thank I. Hlynka for suggesting this problem and to acknowledge gratefully the financial support given by the National Research Council of Canada.

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A NOTE ON A RAPID METHOD FOR THE DETERMINATION OF LIPIDS IN BREWING ADJUNCT CEREALS[1]

GERHARD J. HAAS[2] AND ALAN I. FLEISCHMAN

This note presents some studies on a rapid and reproducible method for the determination of lipids in brewing adjunct cereals. The study is of particular interest because of the extensive collaborative work done in recent years in search of an improved lipid extraction procedure (1,2).

The usual method for the determination of lipids in brewing adjunct cereals involves a 6-hour Soxhlet extraction of the ground sample with low-boiling petroleum ether (30°–60°C.), followed by the evaporation of the solvent and drying of the extracted liquids under standardized conditions (3). In 1959, the Subcommittee on Cereal Adjunct Analysis of the American Society of Brewing Chemists recommended that the extraction time be reduced to 2 hours, and this recommendation has been included in the official methods of the Society (4). This shorter method, while it is an obvious improvement, still requires the use of ground samples and takes approximately 4 hours to complete.

West and Lautenbach (6) recommended the use of a simple column for the extraction employing low-boiling petroleum ether or diethyl ether as the extractant. This procedure simplified the apparatus and simultaneously reduced the extraction time to 3 hours. It retained the disadvantage of requiring a preground sample and, in addition, suffered from the difficulty of using relatively large volumes of flammable extractant.

The Subcommittee on Cereal Adjunct Analysis attempted to use a modified Waring Blendor, in which the sample was extracted by grinding with solvent twice for 2 and 3 minutes, respectively (1). It was found that the range of values obtained for any given sample was rather large. For one sample of corn grits for which the mean value of lipid content was 1.01%, the range in one case was ± 0.28% and in the duplicate set was ± 0.43%.

The method reported here reduces the extraction time to 2.5 minutes without pregrinding and employs the Servall Omnimixer.[3]

A 10-g. unground sample is placed in the metal chamber of the Omnimixer, covered with redistilled low-boiling (30°–60°C.) petro-

[1] Manuscript received April 4, 1960. Contribution from Liebmann Breweries, Brooklyn, New York.
[2] Present address, the Desitin Chemical Company, Providence, R. I.
[3] Ivan Servall, Inc., Norwalk, Conn.

leum ether (75–100 ml.), and extracted by grinding for 2.5 minutes at a potential of 90 volts. During grinding, the cup is immersed in a cold-water bath. The extract is gravity-filtered into a tared receiving flask, and after the residue has been washed onto the filter with small portions of petroleum ether, the solvent is evaporated from the open flask on a hot-water bath. The extracted oils are dried at 103°–105°C. for 75 minutes, cooled to room temperature in a desiccator over calcium chloride, and weighed to the nearest mg.

Complete extraction was routinely effected within 2.5 minutes, and in some cases within 1 minute.

Several samples of adjuncts were analyzed and excellent replication was obtained (Table I).

TABLE I

DETERMINATION OF OILS IN REPLICATE SAMPLES OF ADJUNCT CEREALS BY THE 2.5-MINUTE OMNIMIXER EXTRACTION PROCEDURE

ADJUNCT	NUMBER OF REPLICATE SAMPLES	OIL (Mean Value)	RANGE
		%	%
Rice	4	0.70	± 0.03
Corn grits	3	0.51	± 0.01
Corn flakes A	3	0.53	± 0.01
Corn flakes B	3	0.59	± 0.02

The sample of corn flakes, B, was also assayed in triplicate by the 6-hour Soxhlet extraction method. The mean value found was 0.57% oil, and the range was ± 0.06%.

Drying time of the extracted oils was standardized at 75 minutes. Prolonged drying gave erratic results. Seven 10-g. subsamples of corn flakes and rice were extracted in the Omnimixer and dried for

TABLE II

RATE OF DRYING OF OILS FROM RICE AND CORN FLAKES IN AN ALCONAP AIR-CONVECTION OVEN AT 104°C. AND IODINE UPTAKE BY THE DRIED OILS

DRYING TIME	RICE		CORN FLAKES	
	Oil Extracted	Iodine Absorbed[a]	Oil Extracted	Iodine Absorbed[a]
minutes	*g*		*g*	
0	..	0.63	....	0.52
30	0.120	0.63	0.099	0.52
45	0.101	0.63	0.097	0.52
60	0.085	0.64	0.062	0.51
75	0.082	0.63	0.062	0.51
90	0.087	0.44	0.055	0.31
120	0.098	0.36	0.064	0.12

[a] Iodine uptake is expressed in milli-equivalents per 10 g. of original sample.

various periods of time at 104°C. in an Alconap air-convection oven. The residual oils were weighed after cooling in a desiccator over calcium chloride for 20 minutes. The mass of the residual oil decreased for approximately the first 75 to 90 minutes of drying time and then started increasing (Table II).

The extent of oxidation of the extracted oils was determined by iodine uptake (8). Concomitantly with the mass increase of the extracted oil, the iodine uptake decreased. Even in the case of corn oil where there was a further decrease in the mass of the oil from 75 to 90 minutes, there was already a decided decrease in the iodine uptake after 75 minutes. This indicates that after 75 minutes' drying, some oxidation occurs which brings about an increase in weight.

Moisture did not interfere in the drying experiment. Several freshly extracted oils were analyzed for moisture by the Karl Fischer method (5), and no water was present.

The mass loss during the first 75 minutes of drying time is, apparently, a loss of residual solvent and, possibly, of some other volatile substances. While solvent is present, the temperature of the sample is relatively low. As the sample temperature increases, oxidation sets in.

The Omnimixer and the 6-hour Soxhlet extraction procedure were compared for rice, corn flakes, and corn grits. The Wilcoxon matched-pairs, signed-ranks test (7) was applied to the results to determine whether a difference could be demonstrated between the two methods at the 1%-significance level. The results are shown in Table III.

Wilcoxon showed that the T-statistic, a rank measure, must be equal to or numerically smaller than a tabulated critical value in order to demonstrate a significant difference between two sets of results at any specified level of significance. The magnitude of the critical T-statistic depends upon the number of sample pairs (n).

TABLE III

A Statistical Evaluation of the Results Obtained by the 2.5-Minute Omnimixer Extraction and the 6-Hour Soxhlet Extraction of Cereal Adjuncts by the Wilcoxon Procedure

Cereal	Number of Samples	Wilcoxon T-value	
		Calculated	Critical Value[a]
Rice	22	70.5	49
Corn flakes	9	12.5	2
Corn grits	8	14.0	0

[a] Critical value at 1% level of significance.

From Table III, the computed T-statistic is greater than the critical T-statistic, and, therefore, no difference between the two methods was demonstrated at the 1% level of significance.

Some comparative determinations on corn and rice by the Omnimixer method, the 6-hour Soxhlet extraction procedure, and the method of West and Lautenbach (6) are shown in Table IV.

TABLE IV

COMPARISON OF RESULTS FOR OIL CONTENT OF CORN AND RICE SAMPLES IN THREE PROCEDURES

Sample	Oils		
	Omnimixer[a]	Column	Soxhlet[b]
	%	%	%
Corn 1	0.57	0.59	0.61
2	0.60	0.60	0.57
3	0.60	0.60	0.55
4	0.51	0.45	0.46
5	0.71	0.66	0.64
Rice 1	0.93	1.02	1.00
2	0.65	0.63	0.66
3	0.96	0.90	0.96
4	0.84	0.77	0.82
Wilcoxon-T[c]		8.5	12.5
Critical-T		2.0	2.0

[a] Extraction for 2.5 minutes.
[b] Extraction for 6 hours.
[c] T-values determined by comparing the Omnimixer method with each of the other methods.

The Wilcoxon test (7) failed to demonstrate a difference between the results obtained by the three methods at the 1%-significance level.

With the Omnimixer method, the use of diethyl ether was compared with that of petroleum ether as an extractant for both rice and corn. On application of the Wilcoxon test (7) to the results of an experiment in which seven samples were used, no difference could be found in the results with either extractant at the 1%-significance level.

Acknowledgment

The technical assistance of Peter Liebmann, Alex Kuvish, and Sam Vento is gratefully acknowledged. Thanks are also due to John Bain for the statistical work.

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NOTE ON A SIMPLIFIED RAPID METHOD FOR DETERMINING ALPHA-AMYLASE ACTIVITY[1]

SVEN HAGBERG[2]

Recently the author described a rapid method for determining the alpha-amylase activity of wheat and rye products (2). A simple modification of this method called the "falling-number" method is described in the present paper.

The apparatus described by Hagberg (2) is used except the electrical flash watch, which is not needed. Seven grams of flour or finely pulverized grain and 25 ml. distilled water (20°C.) are thoroughly shaken by hand in the precision test tube (21 by 220 mm.) using 20 vigorous shakes. The test tube with the stirrer-viscometer in position is immersed in the boiling-water bath and the stopwatch started. The surface of the suspension in the test tube should be about 5 cm. under the surface of the boiling water. Five seconds after immersion of the test tube, the suspension is stirred by moving the stirrer up and down at the rate of 2 times per second. The stirring is continued during 55 seconds and, after exactly 60 seconds from immersion of the tube in the bath, the stirrer-viscometer is allowed to drop by its own weight from its uppermost position. When the stirrer-viscometer has dropped a distance of 70 mm. the watch is stopped. The total time in seconds is the "falling number," which is a measure of the amylase activity.

The "falling number" will vary from 60 seconds or more for wheat and rye flours with high amylase activity (e.g., flour from

[1] Manuscript received July 25, 1960.
[2] Head, Institution of Food Chemistry, Statens Hantverksinstitut, Stockholm 4, Sweden.

sprout-damaged grain) up to 400 seconds or more for flour with low amylase activity.

The ratio of flour to water may be varied. Thus, for flours of low alpha-amylase activity, less than 7.0 g. may be employed. If flours of high alpha-amylase activity are commonly analyzed, the amount of flour may be standardized at a higher level than 7.0 g. per 25 ml. of water.

When the test is correctly performed, the values from two single tests do not deviate by more than ±2.5% of the mean value.

Discussion

The falling-number method gives reliable results over a wide range of alpha-amylase concentrations. The relationships between falling-numbers and alpha-amylase activities expressed as SKB units (1) for several wheat flours are shown in Fig. 1. This close relation-

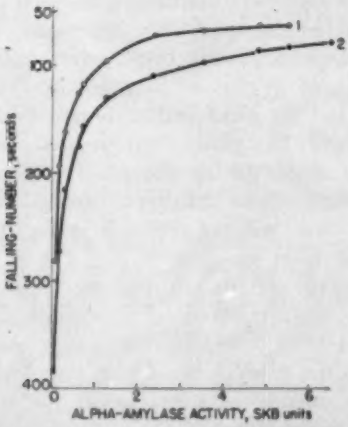


Fig. 1. Relation between falling number and alpha-amylase activity (SKB units) based on the values for several flours. Curve 1 was determined employing a concentration of 7 g. flour per 25 ml. water; curve 2 with 9 g. flour per 25 ml. water.

ship together with the precision and simplicity with which the falling-number can be determined render the procedure valuable where a simple, rapid, inexpensive, and sensitive method is required.

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A NOTE ON THE CONSTRUCTION OF A SMALL-SCALE DOUGH EXPANSION APPARATUS FOR THE ESTIMATION OF WHEAT QUALITY[1]

H. MILLER, A. G. O. WHITESIDE, AND W. KALBFLEISCH

Suitable apparatus for conducting dough expansion tests were described by Miller, Edgar, and Whiteside (1,2). Since these publications were issued, improvements have been made on the dough expansion apparatus and several requests have been received for detailed plans for reproducing this device.

The Expansion Test is essentially a dough-ball gas-retention test for indicating baking strength and was developed as a method for assessing wheat samples in the early stages of the wheat breeding program when only small quantities are available. A dough mixed from 5 g. of flour using a suitable formula is fermented in air before being molded and placed in the expansion apparatus. The expansion of the dough takes place in a weak brine solution and the liquid displacement is measured in the calibrated tube. The apparatus provides a tight seal for the head and a dough holder for supporting the expanding dough. The plastic top enables the operator to note the behavior of the dough during the test. Studies with spring wheats grown in Canada have shown that expansion values are highly correlated with loaf volume, and the test is being used currently for the screening of hybrid lines for quality.

Specific dimensions and details of construction for this dough tester are presented in an engineering drawing, Fig. 1. The metal cup is fabricated from a brass tube, one end being closed off by a circular plate soldered into the tube. A brass ring with a machine-cut thread is soldered to the outside of the tube at the top. The cup and knurled collar are nickel-plated.

The plastic head assembly is fabricated from acrylic plastic, the dome-shaped crown being formed in a compression mold at a temperature of 250°F. A cone-shaped piece of hardwood was cut in a carpenter's wood lathe to serve as a mold for the bottom of the crowned plastic plate; a corresponding piece of wood was used as the top section of the mold. After the plastic plate is formed it is machined to provide a close fit onto the cup and to provide a groove for the neoprene washer. A ring cut from plastic tubing is set into the top

[1]Manuscript received August 30, 1960. Genetics and Plant Breeding Research Institute Contribution No. 42, and Engineering Research Service Contribution No. 3, Canada Department of Agriculture, Ottawa, Ontario.

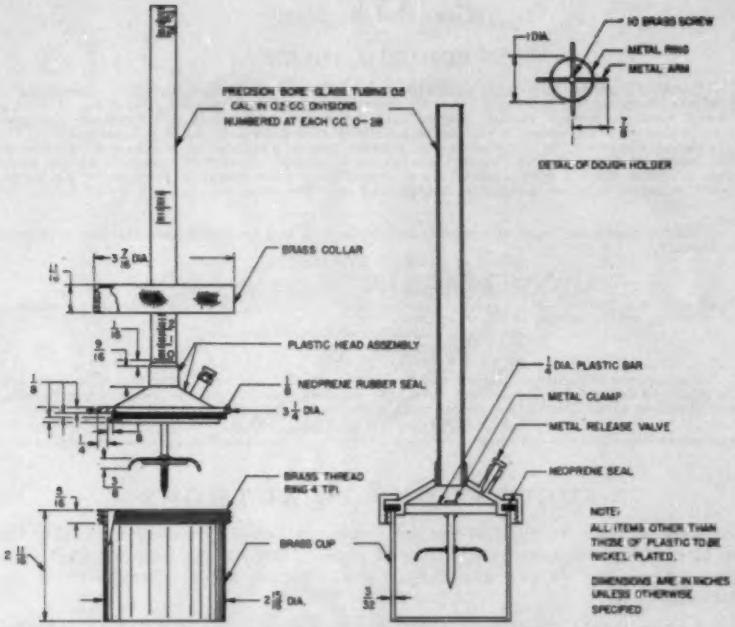


Fig. 1. Engineering drawing of dough expansion apparatus.

to hold the graduated glass tubing which is cemented in place with thermosetting cement. A simple metal relief valve is fitted into the plastic dome to adjust for zero in the graduated tube.

The dough-holding device is made from a threaded screw nail, to which are attached four wing blades and a ring. This, in turn, is mounted on a small plastic bar cemented to the bottom of the plastic head assembly.

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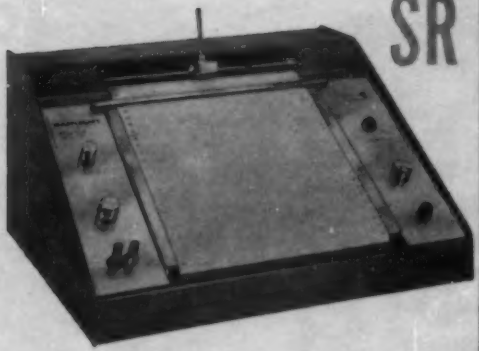
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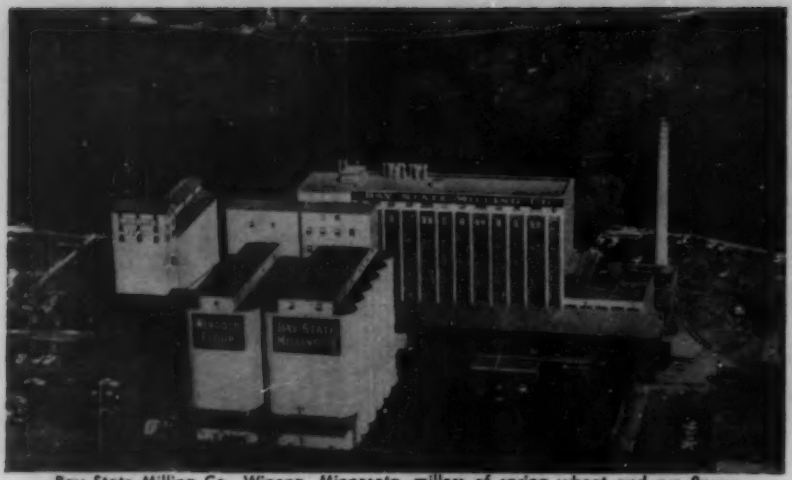
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